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Original Article

Loss of MEG3 contributes to the enhanced migration and invasion in arsenic-induced carcinogenesis through NQO1/FSCN1 pathway

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Abstract: Arsenic ranks at the top among all toxic metals and poses a serious threat to human health. Inorganic arsenite and arsenate compounds have been classified as human carcinogens in various types of cancers. Maternally expressed gene 3 (MEG3), a tumor suppressor that is commonly lost in cancer, was investigated in this study for its role in the migration and invasion of arsenic-transformed cells. Our results showed that MEG3 was downregulated in both arsenic-transformed cells (As-T) and cells treated with low doses of arsenic for three months (As-treated). The analysis using TCGA dataset revealed that MEG3 expression was significantly reduced in the tumor tissues from human lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) compared to normal lung tissues. The results from the methylation-specific PCR (MSP) assay demonstrated enhanced methylation in the MEG3 promoters in both As-T and As-treated cells, indicating that increased methylation of the MEG3 promoter caused MEG3 downregulation in these cells. Moreover, As-T cells displayed increased migration and invasion and higher levels of NAD(P)H quinone dehydrogenase 1 (NQO1) and fascin actin-bundling protein 1 (FSCN1). Consistently, the results from immunohistochemistry staining showed that both NQO1 and FSCN1 are highly expressed in human lung squamous cell carcinoma tissues compared to those in normal lungs. Knockdown of MEG3 in normal BEAS-2B cells also led to increased migration and invasion, along with elevated levels of NQO1 and FSCN1. The negative regulation of MEG3 on FSCN1 was restored by NQO1 overexpression in both As-T and BEAS-2B cells. The results from immunoprecipitation assays confirmed the direct binding of NQO1 to FSCN1. Overexpression of NQO1 increased migration and invasion abilities in BEAS-2B cells, while knockdown of NQO1 by its shRNA reduced these two hallmarks of cancer. Interestingly, the reduced migration and invasion by NQO1 knockdown were restored by FSCN1. Collectively, the loss of MEG3 upregulated NQO1, which in turn stabilized FSCN1 protein through its direct binding, resulting in elevated migration and invasion in arsenic-transformed cells.

Keywords: Arsenic, MEG3, migration, invasion, NQO1, FSCN1

Introduction

Inorganic arsenic compounds are highly carcinogenic and classified as Group I carcinogens by the International Agency for Research on Cancer (IARC). According to the Agency for Toxic Substances and Disease Registry (ATSDR), arsenic is the most toxic among all toxic metals. Unfortunately, even today, high levels of naturally occurring arsenic in groundwater are still affecting the health of millions of people worldwide [1]. Environmental arsenic exposure may come from air, food, and drinking water. Long-term exposure to arsenic can cause skin lesions and numerous cancers [2, 3]. Depending on the exposure route, arsenic exposure causes cancers of the bladder, skin, lungs, digestive tract, liver, and kidney. In recent decades, arsenic exposure has also been associated with cardiovascular disease and diabetes [4, 5]. Exposure to arsenic during in-utero and early childhood stages has been shown to cause cognitive development delays and increase the risk of premature death in young adults [6, 7].

Maternally expressed gene 3 (MEG3) is an imprinted gene located within the DLK1-MEG3 locus on human chromosome 14q32.3. The MEG3 gene encodes a long noncoding RNA (lncRNA) that is expressed in normal tissues

but significantly downregulated or lost in tissues from various human cancers [8]. Several hypotheses have been proposed to illustrate the reduced expression of MEG3, including promoter hypermethylation [9, 10] and hypermethylation of intergenic differentially methylated regions [11]. Studies have shown that ectopic expression of MEG3 in transformed cells can inhibit cell proliferation [12, 13], cellular invasion/migration [14, 15], angiogenesis [16, 17], and anchorage-independent growth [18] in various types of cancer models. MEG3 is not only a tumor suppressor but also synergizes with other tumor suppressors to augment the repressive effect on tumor growth. For instance, it has been reported that MEG3 can activate p53 by reducing MDM2 levels, thereby stimulating the transcription of specific p53-target genes [19, 20]. Additionally, programmed death ligand-1 (PD-L1) was revealed to be an unappreciated tumor suppressor in aggressive endometrial cancer (EC) cells, and MEG3 was an upstream regulator of PD-L1 [21]. The above evidence indicated the tumor suppressor roles of MEG3 are displayed at different levels of cancer regulatory system, which makes it a master anti-tumor regulator.

NAD(P)H quinone dehydrogenase 1 (NQO1) is a cytoplasmic two-electron reductase that plays a critical role in reducing quinone to hydroquinone, which minimizes the production of reactive oxygen species (ROS). Higher NQO1 expression has been associated with lower levels of superoxide and singlet oxygen, indicating its protective role against oxidative stress [22]. Studies have reported that NQO1 can scavenge superoxide directly, although not as efficiently as superoxide dismutase (SOD) [23]. However, as an additional superoxide scavenger, NQO1 becomes more essential when SOD expression is repressed such as in cardiovascular tissue, where NQO1 would compensate for the relatively low SOD and protect cardiovascular cells from damages induced by superoxide [24]. Given the multiple functions of preventing cells from oxidative stress, NQO1 is usually upregulated in cancer cells as a self-motivated response to enhance anti-tumor activity. The anti-tumor function of NQO1 has recently been emphasized again by NQO1's new role of being a gatekeeper of 20s proteasome, which mediates ubiquitin-independent proteasome degradation of selected tumor suppressors, including p53, p73a, and p33 [25, 26]. However, the protein-binding function of NQO1 does not always make it a tumor suppressor but sometimes renders it a tumor promoter. It has been shown that high NQO1 expression correlates with increased HIF-1α protein levels and poor colorectal cancer survival, whereas NQO1 knockdown in human colorectal and breast cancer cells repressed HIF-1α signaling and slowed tumor growth [27]. In addition to binding with proteins, NQO1 can directly bind to mRNA and regulate its transcription. A recent study of ribonucleoprotein immunoprecipitation has demonstrated that NQO1 binds to a subset of mRNAs in HepG2 cells, such as SERPINA1, which encodes serine protease inhibitor a-1-antitrypsin (A1AT) associated with hepatocellular carcinoma and some other diseases [28].

Fascin actin-bundling protein 1 (FSCN1), is an actin-binding protein that organizes actin filaments into parallel bundles. FSCN1 is found to be expressed in mammalian endothelial, neuronal, and mesenchymal cells, which plays a vital role in the formation of cell protrusions, cell motility, and migration [29, 30]. FSCN1 was reported to promote epithelial-mesenchymal transition (EMT) via increasing Snail1 in ovarian cancer cells [31]. The mounting experimental studies showed that FSCN1 mRNA translation was regulated by many different microRNAs and lncRNAs, such as miR-200b, miR-133b, miR-145, and ZEB1-AS1 [32-35]. Besides, FSCN1 expression was also regulated by cAMP response element-binding protein (CREB) and Wnt/beta-catenin signaling pathways [30, 36]. Due to its tight connection with cell motility, excessive FSCN1 expression is believed to be a hallmark of enhanced migration or invasion. FSCN1 expression and its single nucleotide polymorphisms (SNPs) are regarded as the biomarkers for the progression and prognosis of various types of cancer in the clinic [37, 38], enabling FSCN1 as a prominent therapeutic target [30, 39].

Materials and methods

Reagents

Arsenic trioxide (As_2O_3) was purchased from Sigma (St Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM) and SuperScript first-strand synthesis kit were purchased from Thermo Fisher Sci (Waltham, MA, USA). Trans-

fection reagent PolyJet was purchased from SignaGen Laboratories (Rockville, MD, USA). Primary antibodies against NQO1, FSCN1, DNMT3α, and Protein A/G plus-agarose were purchased from Santa Cruz Biotechnology Inc (Dallas, TX, USA). Primary antibody against SOD2 and secondary antibodies against mouse and rabbit were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against DNMT1, DNMT3β, and GAPDH were purchased from GeneTex Corporation (Irvine, CA, USA). PowerUP SYBR green master mix was purchased from Applied Biosystems (Waltham, MA). ECF substrate for Western blot was purchased from GE Healthcare (Pittsburgh, PA, USA). DNeasy Blood&Tissue kit, EpiTect Bisulfite kit, and EpiTect MSP kit were purchased from Qiagen (Germantown, MD, USA).

Plasmids

The full-length human MEG3 sequences (NR_002766) were synthesized and subcloned into the pEGFP-C1 vector (Clontech, Palo Alto, CA, USA) as described previously [10]. MEG3 shRNA and its control vector were kindly provided by Dr. Shau-Ping Lin (Institute of Biotechnology, National Taiwan University, Taipei, Taiwan). NQO1 overexpression plasmid and its control vector were purchased from Addgene (Cambridge, MA, USA); shRNA targeting NQO1 and its control vector were purchased from OriGene (Rockville, MD, USA). FSCN1 ORF cDNA and its control vector were purchased from GeneCopoeia (Rockville, MD, USA).

Cell culture and stable cell lines

Human bronchial epithelial cells (BEAS-2B) were from ATCC and were cultured in DMEM medium with 10% FBS. For the establishment of stable expression cells, the cells were transfected with 2 μg plasmid DNA in each well of a six-well plate followed by antibiotics selection for at least one month. The verification of gene expression was carried out by immunoblotting or real-time qPCR analysis.

As(III)-transformed cells

BEAS-2B cells were continuously exposed to low doses of As_2O_3. The cells were split twice a week and fresh culture media was added. After three months of exposure, RNA/DNA/protein lysates were isolated from these cells. After six months, the soft agar assay was performed. Single colonies from soft agar were picked up and expanded in tissue culture. These cells were considered As(III)-transformed cells. Passage-matched BEAS-2B cells without As(III) exposure were used as control.

Immunoblotting analysis

The cells were cultured in 6-well plates. After reaching 90% confluence, the cells were washed with PBS and then lysed using a boiling buffer. The whole-cell lysates were sonicated after heating (99°C for 5 min). Protein concentrations were measured. Proteins were separated by SDS-PAGE gels followed by incubation with primary antibodies overnight. The blots were then probed with secondary antibodies. Proteins were visualized using ECF substrate.

Real-time qPCR

RNA was extracted and purified using Qiagen RNeasy mini kit. cDNA for MEG3 and NQO1/FSCN1 mRNA were synthesized using a SuperScript first-strand synthesis kit. Primers were designed using Primer-Blast with forward (F) and reverse (R) sequences as followed:

	Reverse	Forward
MEG3	GAGCGAGTCAGGAAGCAGT	GGCATGGACGAGCTGTACAAG
NQO1	ATAAGCCAGAACAGACTCG	GAAGAAAGGATGGGAGGTG
FSCN1	GTGTGGGTACGGAAGGCAC	CCAGGGTATGGACCTGTCTG

Levels of MEG3 and mRNA of NQO1 and FSCN1 were measured using PowerUp SYBR Green master mix and GAPDH as a control. The value of the cycle threshold (CT) was examined. Data were analyzed by calculation of ΔΔCT.

Migration and invasion assays

The invasion assay was conducted using the Biocoat Matrigel Invasion Chambers (Corning, NY, USA) according to the manufacturer's instructions. Migration assay was conducted using chamber inserts (Corning, NY, USA). Cells were seeded in the chamber inserts at a density of 1.0-2.0 × 10^4 per well of 24-well plate in 500 μL medium (0.1% FBS). The inserts were placed into the wells with 700 μL culture medium (10% FBS). After 24 hours, the cells were washed with PBS, fixed with 3.7% formalin and methanol followed by Giemsa staining. The images were captured using an Olympus microscope. The number of invaded and migrated cells were counted and recorded.

Immunoprecipitation

Cells were lysed in cell lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM Na_3VO_4, 0.5% NP-40, and complete protein cocktail inhibitors from Roche) on ice. Lysate (0.5 mg) was precleared by incubation with Protein A/G plus-agarose and then incubated with the specific antibody at 4°C for overnight. Protein A/G plus-agarose (20 µL) was added to the mixture and incubated with agitation for an additional 4 hours at 4°C. The immunoprecipitants were washed three times with cell lysis buffer and then subjected to Western blot analysis.

Methylation-specific PCR (MSP)

Genomic DNA from the cells was first extracted using the DNeasy Blood & Tissue kit according to the product instruction. Then the bisulfite conversion of genomic DNA was performed using EpiTect Bisulfite kit following the manufacturer's protocol. The methylation statuses of *Meg3* promoter were evaluated by methylation-specific polymerase chain reaction (MSP) using EpiTect MSP kit. PCR amplification was performed using the following cycling conditions: 95°C 15 min; 94°C 30 s, 70°C 30 s, 72°C 30 s, 5 cycles; 94°C 30 s, 65°C 30 s, 72°C 30 s, 5 cycles; 94°C 30 s, 60°C 30 s, 72°C 30 s, 30 cycles; 72°C 7 min. The PCR products were separated on a 3% agarose gel with ethidium bromide and visualized under UV illumination. The primers for the methylated (M) reaction were as follows: 5'-GTT AGT AAT CGG GTT TGT CGG C (sense) and 5'-AAT CAT AAC TCC GAA CAC CCG CG (antisense), with a 160-bp amplification product. The primers for the unmethylated (U) reaction were as follows: 5'-GAG GAT GGT TAG TTA TTG GGG T (sense) and 5'-CCA CCA TAA CCA ACA CCC TAT AAT CAC A (antisense), with a 120-bp amplification product [40].

Immunohistochemistry staining (IHC)

The human lung tissue slides used in IHC staining were all obtained from surgical resection provided by the Center for Biospecimen Research & Development NYU Langone Health. The control sample was from a 74-year-old White female. The human lung squamous carcinoma samples LUSC1, LUSC2, and LUSC3 were from a 90-year-old White male, a 70-year-old male whose ethnicity is not listed, and a 78-year-old White female, respectively. More detailed human sample information, such as smoking status and medical history, is enclosed in Supplementary Table 1. The lung tissues were fixed and embedded in paraffin, and thin sections (5 µM) were cut from the tissue blocks. The sections were then mounted on a slide and subjected to deparaffinization, rehydration, and antigen retrieval. The primary antibody was then applied to the section slides and allowed to bind to the target protein. To visualize the bound antibody, a secondary antibody conjugated to an enzyme was applied. The resulting complex was then visualized under a Nikon microscope, and the intensity of staining (brown color) indicates the presence and distribution of the target protein in the tissues.

Statistical analysis

The student's t-test was used to evaluate the difference between different groups. A value of $P < 0.05$ was considered significant.

Results

Arsenic-transformed (As-T) cells exhibit enhanced abilities for both migration and invasion

Cancer progression is characterized by the migration and invasion of cancer cells, which are critical factors in the development of metastasis. To assess these processes, we employed transwell and wound healing assays, which demonstrated that both migration and invasion were significantly increased in As-T cells compared to passage-matched normal BEAS-2B (As-M) cells (**Figure 1A-C**). The tumor suppressor MEG3 is known to be repressed in human lung cancer tissues [13], suggesting that its downregulation may play a role in the malignancy of arsenic-transformed cells. This coincidence motivated our investigation into the potential importance of MEG3 downregulation in this context, given its critical role as an upstream regulator of multiple cellular molecules.

Increased methylation of MEG3 promoter causes its downregulation in both arsenic-transformed and arsenic-treated cells

In arsenic-transformed human lung epithelial (BEAS-2B) cells (As-T), MEG3 expression was

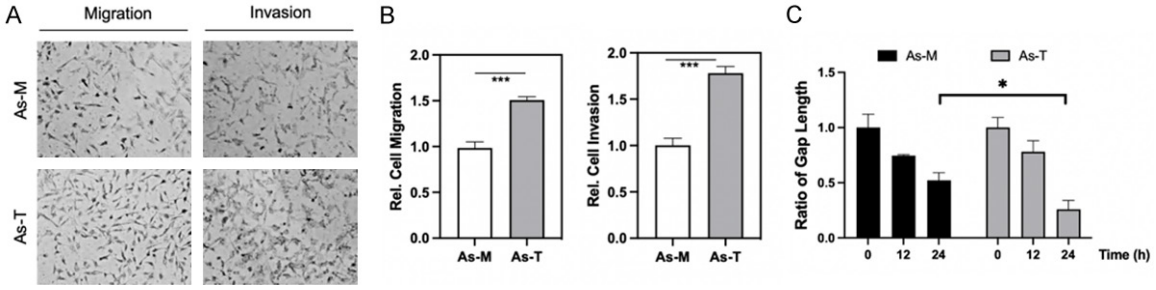

Figure 1. Arsenic-transformed cells exhibit enhanced abilities of both migration and invasion. Normal human bronchial epithelial (BEAS-2B) cells were chronically exposed to low-dose arsenic for over six months. The soft agar assay was performed and single colonies from soft agar were isolated and expanded in the culture plates. These cells were used as arsenic-transformed cells (As-T), and passage-matched normal BEAS-2B cells without arsenic exposure were used as control (As-M). (A) Cell migration and invasion were examined by transwell assay. The images represent one of three images captured in each group. (B) Quantifications of the migrated and invaded cell numbers of the transwell assay above. (C) Cell migrating speeds were measured by wound healing assay, and the ratios of the gap lengths at 0, 12, and 24 hours to the gap length at 0 hours were quantified and compared. * and ***, $p<0.05$ and $p<0.001$, respectively.

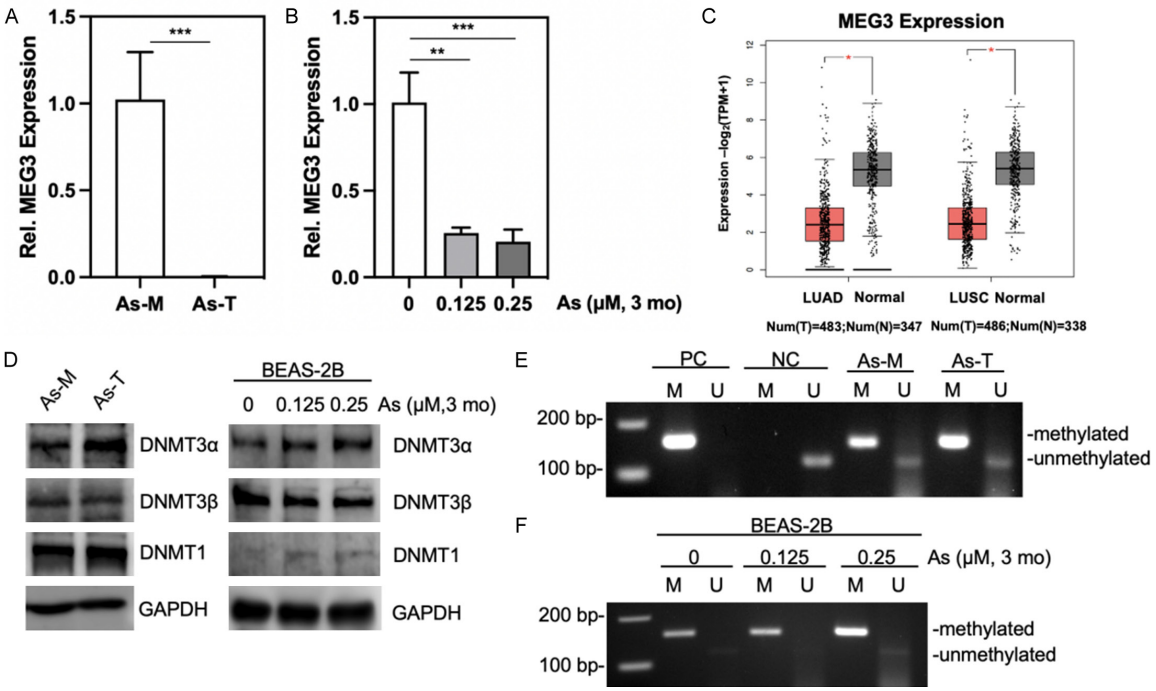

Figure 2. Increased methylation of MEG3 promoter causes its downregulation in both arsenic-transformed and arsenic-treated cells. Wild-type BEAS-2B cells were treated with 0.125 μM and 0.25 μM arsenic for three months. RNA/DNA/protein lysates were collected. (A and B) RNAs from the As-M and As-T cells and the arsenic-treated cells were harvested and MEG3 levels were measured using qRT-PCR. (C) MEG3 expression levels from human lung adenocarcinoma (LUAD) and human lung squamous cell carcinoma (LUSC) tissues were analyzed and compared to the normal tissues using the TCGA database. (D) The As-M and As-T cells, together with arsenic-treated cells were harvested for immunoblotting analysis. (E and F) The methylation statuses of MEG3 promoters were analyzed by methylation-specific PCR in As-M, As-T, normal BEAS-2B cells treated with 0.125 and 0.25 μM arsenic. The positive control (PC) yielded a methylated band at 160 bp and the negative control (NC) yielded an unmethylated band at 120 bp. *, **, and ***, $p<0.05$, $p<0.01$, and $p<0.001$, respectively.

almost lost compared to that in passage-matched normal BEAS-2B cells (As-M) (**Figure 2A**). The expression of MEG3 was also reduced in cells exposed to arsenic at 0.125 and 0.25 μM for three months in a dose-dependent manner (**Figure 2B**). We have analyzed the MEG3 expression levels using the TCGA database, and the results showed that MEG3 was mark-

edly downregulated in human lung adenocarcinomas (LUAD) and lung squamous cell carcinomas (LUSC) compared to the normal human lung tissues (**Figure 2C**).

It has been reported that the downregulation of MEG3 in nickel-exposed cells was due to the increased methylation of the MEG3 promoter by DNMT3β, thus preventing the transcription initiation complexes from recognizing the MEG3 promoter and starting transcription [10]. It was also observed that hypermethylation of the MEG3 promoter correlates with the inactivation of MEG3 in retinoblastoma patients [41]. Hereby, we examined the three most common DNA methyltransferases in humans and found that DNMT3α increased significantly in cells exposed to arsenic for three months and As-T cells (**Figure 2D**). To test whether promoter methylation of MEG3 is the cause for its downregulation, methylation-specific PCR (MSP) was carried out. The results showed that methylation was increased (the methylated band at 160 bp) in the As-T cells and in cells exposed to arsenic at 0.125 μM and 0.25 μM for 3 months (**Figure 2E**, **2F**). Those data indicated that the increased DNMT3α expression in As-T and arsenic-exposed cells facilitated MEG3 methylation at its promoter region, resulting in the repression of MEG3 expression.

MEG3 reduces migration and invasion of arsenic-transformed cells

The results from **Figures 1** and **2** showed enhanced migration and invasion and loss of MEG3 expression in the As-T cells, respectively. To study whether the downregulation of MEG3 contributed to the enhanced migration and invasion in As-T cells, we established As-T cells with stable expression of MEG3. The results from qRT-PCR validated the ectopic expression efficiency (**Figure 3D**). The results from migration and invasion assays showed that the overexpression of MEG3 significantly reduced the migration and invasion of As-T cells (**Figure 3A-C**), indicating that MEG3 negatively regulates the migration and invasion of arsenic-transformed cells.

NQO1 and FSCN1 are upregulated in both arsenic-transformed cells and lung tissues from human lung squamous cell carcinoma

Our study found that NQO1 and FSCN1 were upregulated in both arsenic-transformed cells and cells exposed to arsenic for three months (**Figure 4A**). NQO1 has a multifunctional role in regulating protein stability and responding to oxidative stress, and it is frequently elevated in various cancer types. FSCN1, on the other hand, is a structural protein known to increase cell motility. The simultaneous increase of NQO1 and FSCN1 in arsenic-transformed cells led us to hypothesize that their overexpression may contribute to the enhanced migration and invasion observed. Squamous cell carcinoma is the most common type of lung cancer associated with arsenic exposure [42], we further examined NQO1 and FSCN1 expression levels in the lung tissues from three patients diagnosed as lung squamous cell carcinoma (LUSC) and one normal human lung (control) using immunohistochemistry (IHC) staining assay. Our results showed that NQO1 and FSCN1 were consistently overexpressed in LUSC patient samples but mildly expressed in epithelial cells around the trachea and bronchus of the normal human lung sample (**Figure 4C**). To aggrandize the reliability, we analyzed the NQO1 and FSCN1 expressions of four different stages of LUSC. In each stage of LUSC, both NQO1 and FSCN1 expression levels were significantly elevated compared to the control (**Figure 4B**).

MEG3 negatively regulates NQO1 and FSCN1 at post-transcriptional level

MEG3, as a universal tumor suppressor, is not surprising to be found to play an inhibitory role in the migration and invasion of As-T cells. To further study the mechanism of how MEG3 inhibits migration and invasion, the expression of several genes related to cell motility was assessed. Results showed that NQO1 and FSCN1 protein levels were increased in the As-T cells compared to those in passage-matched normal BEAS-2B (As-M) cells (**Figure 4A**), and ectopic expression of MEG3 could reverse the enhancements of NQO1 and FSCN1 (**Figure 5A**). To test whether the observation in **Figure 5A** is cell-type specific, the normal BEAS-2B cells with stable overexpression or knockdown of MEG3 were established and validated (**Figure 5B**). NQO1 and FSCN1 were downregulated or upregulated in BEAS-2B cells with stable MEG3 overexpression or knockdown, respectively (**Figure 5C**). The results from RT-qPCR showed that the mRNA levels of both NQO1 and FSCN1 remained similar in As-T cells and in As-M cells (**Figure 5D**) and in the BEAS-2B cells

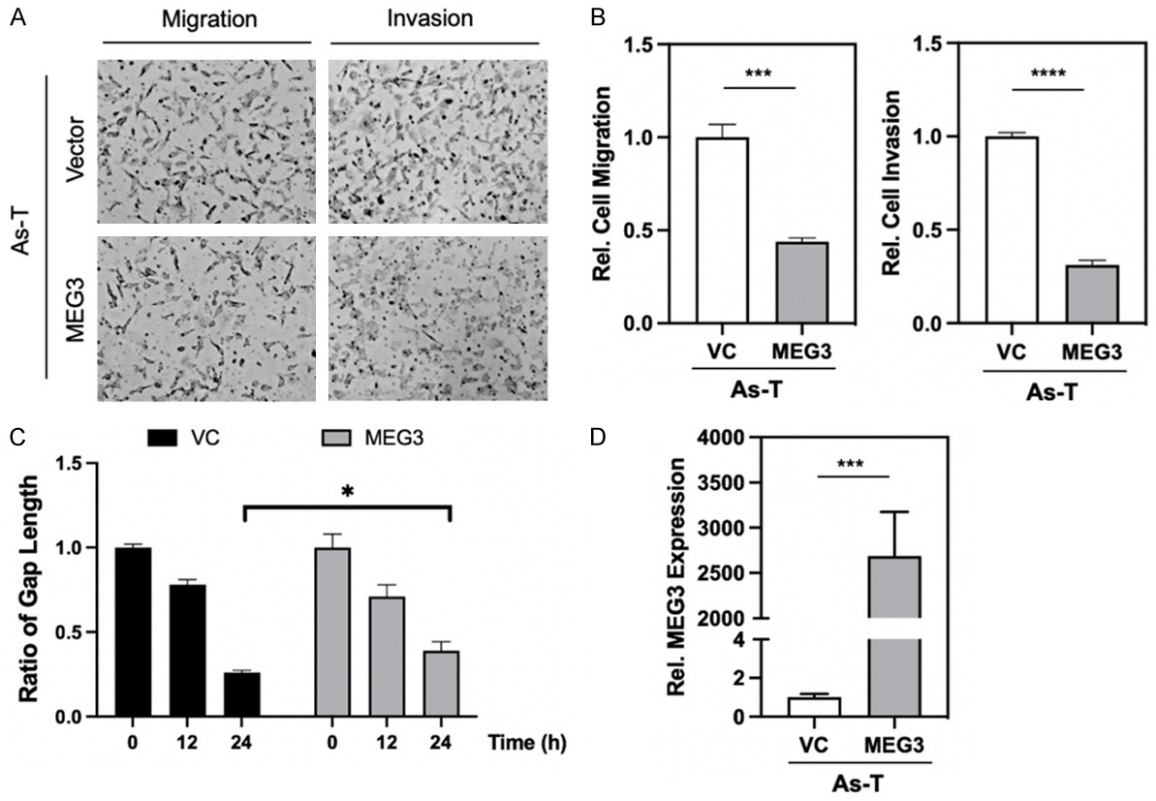

Figure 3. MEG3 reduces migration and invasion of arsenic-transformed cells. MEG3 overexpression plasmid and its control vector were delivered into the As-T cells followed by antibiotics selection to obtain MEG3 stably expressed cells. (A) Migration and invasion in As-T with or without stable expression of MEG3 were examined using the transwell assay. The images were captured using Olympus microscope and each image represents one of three images captured in each group. (B) Quantification of the migrated and invaded cells presented in (A). (C) Migrating speeds of As-T with or without stable expression of MEG3 were measured by wound healing. The ratios of the gap lengths at 0, 12, and 24 hours to the gap length at 0 hours were quantified and compared. (D) MEG3 expression levels in As-T with or without stable expression of MEG3 were validated using RT-qPCR. *, ***, ****, $p<0.05$, $p<0.001$, $p<0.0001$, respectively.

with or without overexpression or knockdown of MEG3 (**Figure 5E**). Those data suggested that MEG3 regulates NQO1 and FSCN1 at post-transcriptional levels. MEG3 is a master regulator that directly binds to RNA or protein. As reported, MEG3, acting as competing endogenous RNA, binds with miR-27a to prevent the invasion of bladder cancer cells [43]. MEG3 binds with proteins, such as p53a and p73, to maintain their stabilities [20, 25]. To find out how FSCN1 is negatively regulated by MEG3, BEAS-2B cells were treated with cycloheximide (CHX) and MG132, which are inhibitors of protein translation and proteasome-mediated protein degradation, respectively. We found the protein level of FSCN1 was increased when cells were treated with MG132 or CHX (**Figure 5F**). The above data suggested that the downregulation of FSCN1 protein by MEG3 is through post-translational modification.

NQO1 stabilizes FSCN1 through direct binding, thus mediating cell migration and invasion

So far, our results demonstrated that MEG3 negatively regulated NQO1 and FSCN1 and inhibited cell migration and invasion of As-T cells. Nevertheless, it remains unknown whether NQO1 is a mediator of the MEG3-FSCN1 pathway. We have established NQO1 stable knockdown or overexpression BEAS-2B cells. The results from **Figure 6E** showed that the knockdown of NQO1 in BEAS2-B cells reduced the FSCN1 protein level. Overexpression of NQO1 stabilized FSCN1 in BEAS-2B and rescued the reduction of FSCN1 induced by elevated MEG3 (**Figure 6F**). Not surprisingly, the overexpression of NQO1 in normal BEAS-2B cells enhanced their migration and invasion abilities (**Figure 6A**, **6B**). In contrast, the knockdown of NQO1 suppressed the migration and invasion

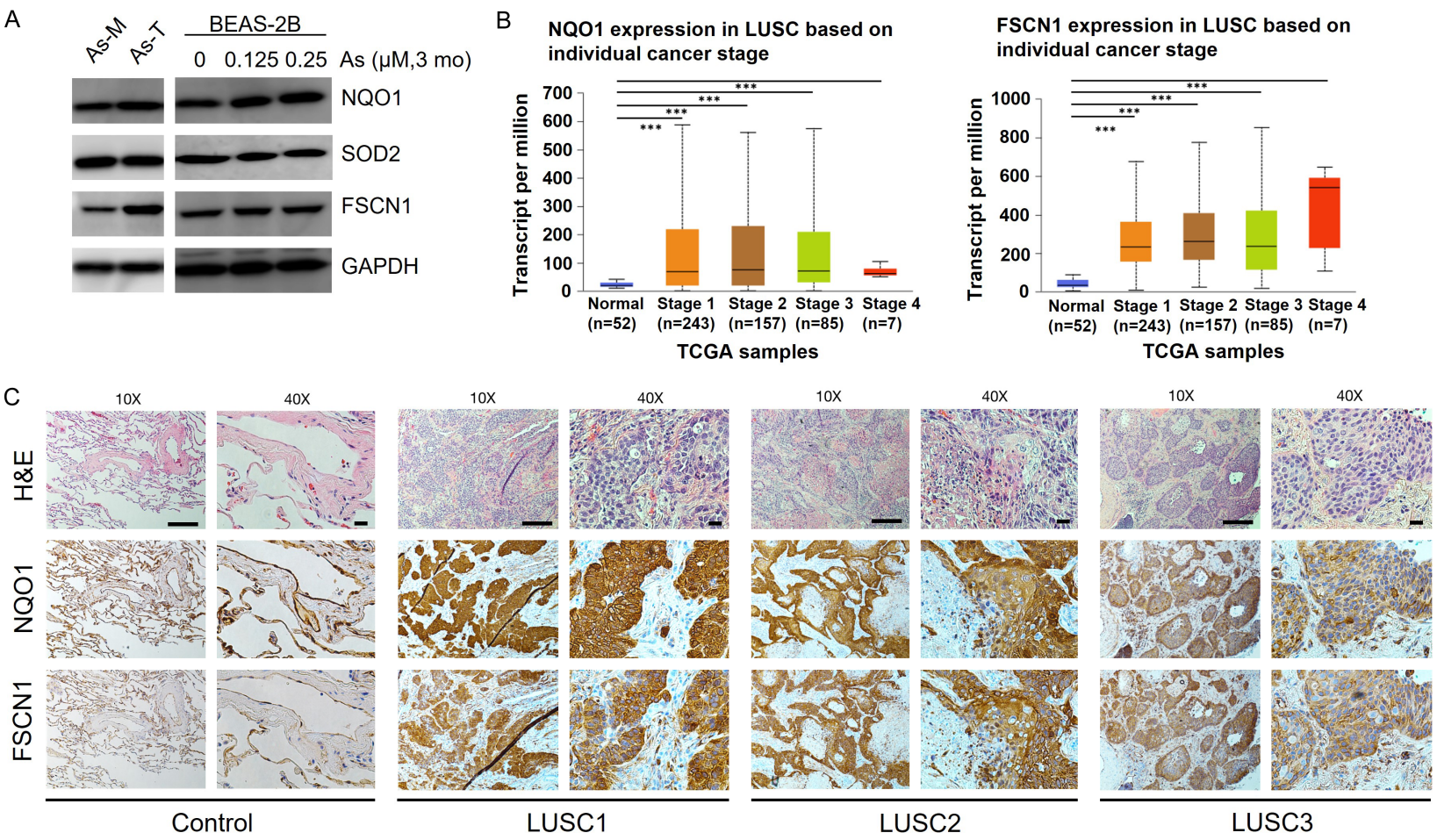

Figure 4. NQO1 and FSCN1 are upregulated in arsenic-transformed cells and lung tissues from human lung squamous cell carcinoma. (A) Whole-cell lysates from As-M, As-T cells, and BEAS-2B cells treated with 0.125 and 0.25 μM arsenic for three months were isolated for immunoblotting analysis. (B) Expressions of NQO1 and FSCN1 were analyzed on each stage of LUSC with patients' information obtained from the TCGA database. (C) Representative H&E and IHC staining specific to NQO1 and FSCN1 of normal human lung tissue (control) and three individual human lung squamous cell carcinoma tissues in 10X and 40X magnification. The scale bars represent 1,000 μm and 100 μm in 10X and 40X views respectively.

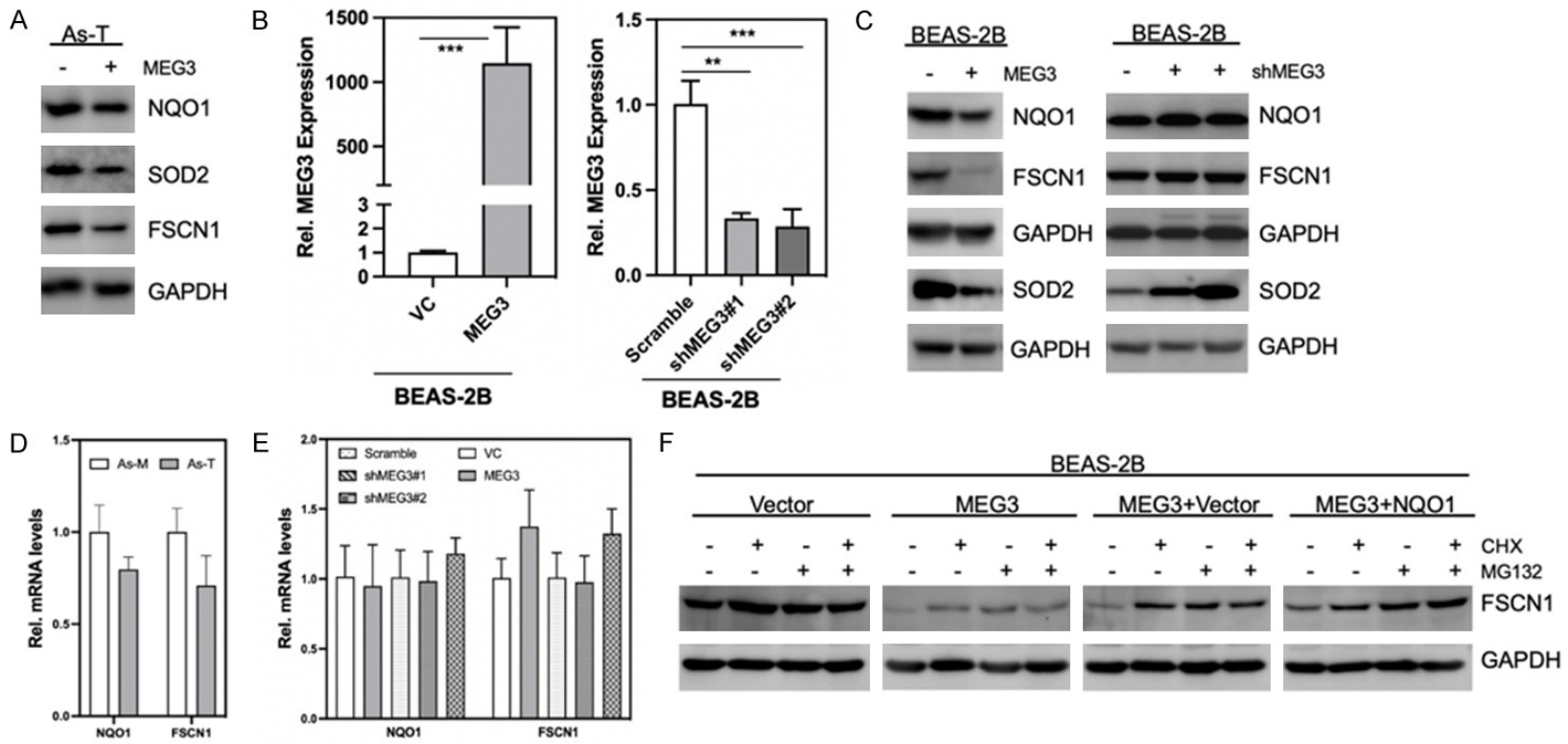

Figure 5. MEG3 negatively regulates NQO1 and FSCN1 at post-transcriptional level. (A) Whole-cell lysates from As-T cells with or without MEG3 overexpression were isolated for immunoblotting analysis. (B) RNAs from BEAS-2B cells with or without MEG3 overexpression of knockdown were harvested for RT-qPCR to measure MEG3 levels. (C) Whole-cell lysates from BEAS-2B cells with or without MEG3 overexpression or knockdown were isolated for immunoblotting analysis. (D and E) NQO1 and FSCN1 mRNA levels were evaluated in the As-M and As-T cells, BEAS-2B cells with or without MEG3 overexpression or knockdown cells using RT-qPCR. (F) The cells with different expressions of MEG3 and NQO1 were treated with cycloheximide, or MG132, or their combination. Whole-cell lysates were harvested to determine FSCN1 protein levels using immunoblotting analysis. ** and ***, $p<0.01$ and $p<0.001$, respectively.

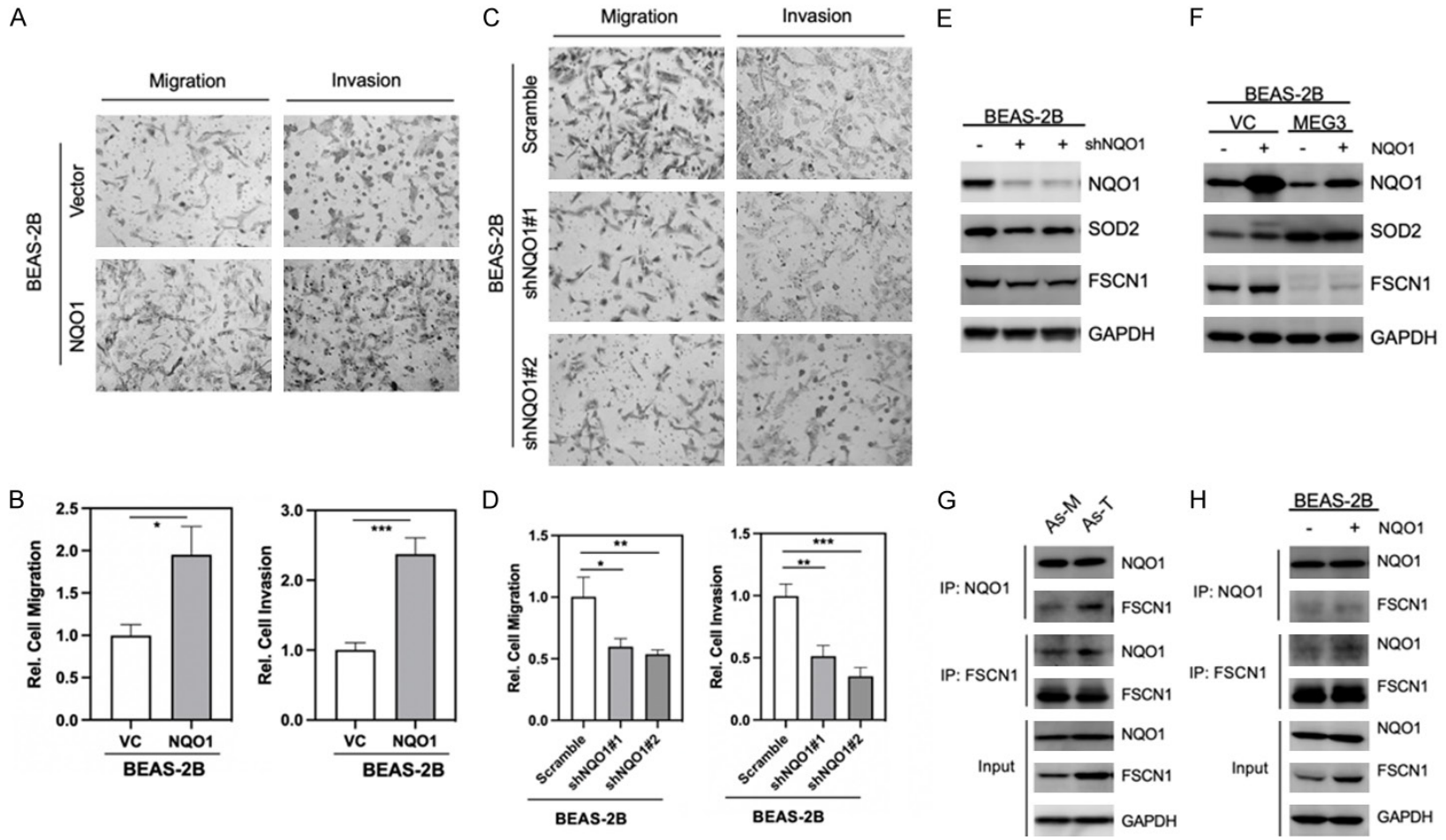

Figure 6. NQO1 stabilizes FSCN1 through direct binding, thus mediating cell migration and invasion. (A and C) Migration and invasion in BEAS-2B with or without NQO1 expression (A) or NQO1 knockdown (C) were evaluated by the transwell assay, images represent one of three images captured in each group. (B) Quantification of the results of (A). (D) Quantification of the migrated and invasive cells of (C). (E) Knockdown of NQO1 was achieved by two different hairpins in normal BEAS-2B cells. The whole-cell lysates from them were collected for immunoblotting analysis. (F) The whole-cell lysates from BEAS-2B cells with the overexpression of MEG3, NQO1, or a combination of both were collected for immunoblotting analysis. (G and H) The immunoprecipitation assay was used to detect the protein-protein interaction between FSCN1 and NQO1. The whole protein (input) and IP lysates were then subjected to immunoblotting analysis. *, **, and ***, $p<0.05$, $p<0.01$, and $p<0.001$, respectively.

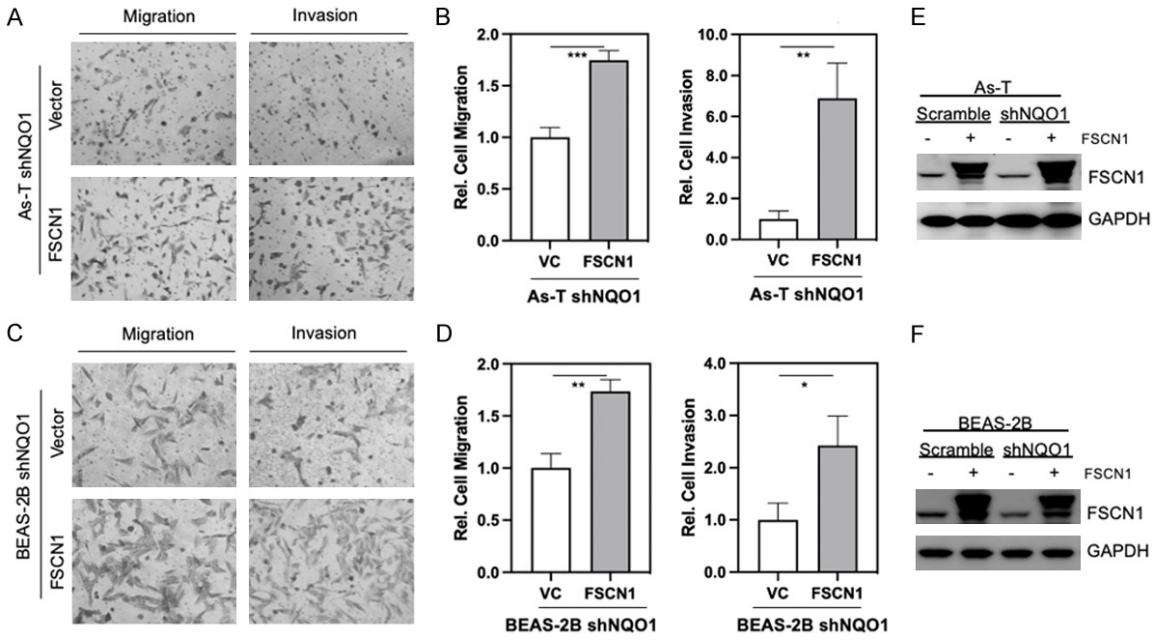

Figure 7. FSCN1 is a downstream target of NQO1 in the regulation of cell migration and invasion. Stable expressing FSCN1 in As-T and BEAS-2B cells with or without NQO1 knockdown were constructed. (A and C) The migration and invasion were evaluated using transwell assay. The images were captured using Olympus microscope. The images represent one of three images captured in each group. (B and D) Quantification of the migrated and invaded cells presented in (A, C) respectively. (E and F) The validation of FSCN1 expression efficiencies by immunoblotting analysis. *, **, and ***, $p<0.05$, $p<0.01$, and $p<0.001$, respectively.

of BEAS-2B cells (**Figure 6C**, **6D**). We observed that the arsenic-transformed cells with stable knockdown of NQO1 are not in healthy condition, leading us to speculate that NQO1 is extremely important for the survival of arsenic-transformed cells. The results from transwell assays indicated that the knockdown of NQO1 significantly impaired the migrative and invasive abilities of As-T cells (Supplementary Figure 1).

NQO1 is known to act as a gatekeeper for proteasomes and can prevent protein degradation by interacting with the protein. To determine if there is a direct interaction between NQO1 and FSCN1, we conducted an immunoprecipitation assay. Our results demonstrated that NQO1 bound to FSCN1 in both As-M and As-T cells, with a higher binding affinity in As-T cells (**Figure 6G**). We further validated our findings by performing the immunoprecipitation assay in BEAS-2B cells with or without NQO1 overexpression. The results revealed that NQO1 overexpression led to increased binding of FSCN1 (**Figure 6H**). These findings suggest that NQO1 may maintain FSCN1 protein stability through direct binding, which may contribute to the enhanced migration and invasion of As-T cells and BEAS-2B cells with NQO1 overexpression.

FSCN1 is a downstream target of NQO1 in the regulation of migration and invasion

As shown in **Figure 6** and Supplementary Figures 1, 2, 3, the knockdown of NQO1 reduced cell migration and invasion in both normal BEAS2-B and As-T cells, while simultaneously decreasing FSCN1 protein expression. To confirm the role of FSCN1 in the enhanced migration and invasion of As-T cells, the FSCN1 expressing vector was transfected into the cells with stable knockdown of NQO1. Their expression levels were verified using immunoblotting analysis (**Figure 7E**, **7F**). The enforced expression of FSCN1 rescued the migration and invasion in As-T and BEAS-2B cells with stable knockdown of NQO1 (**Figure 7A**, **7C**). In conclusion, the downregulation of MEG3 led to the upregulation of NQO1, which directly bound to FSCN1 and promoted enhanced migration and invasion in As-T cells.

Discussion

Arsenic exposure is a significant global public health issue, and inorganic arsenic compounds are categorized as human group I carcinogens. While the mechanisms behind arsenic-induced carcinogenesis have been gradually unveiled over recent decades, a comprehensive understanding of the process remains elusive. An understanding of the molecular mechanisms underlying arsenic carcinogenesis is crucial for both prevention and intervention. Arsenic is widely accepted as a nongenotoxic carcinogen that modifies gene expression without causing direct gene damage. Thus, epigenetic modifications play a dominant role in the gene expression alterations triggered by arsenic exposure. The present study demonstrates that the decreased expression of MEG3 induced by arsenic exposure is the result of increased methylation in its promoter region. Furthermore, our study provides evidence that the epigenetic modification that causes the downregulation of MEG3 is responsible for the enhanced migration and invasion of arsenic-transformed cells through regulating the NQO1/FSCN1 pathway.

MEG3, a long non-coding RNA, has recently gained recognition for its potent anti-tumor activity. Downregulation of MEG3 has been observed in various cancer cells [44] as well as in lung cancer tissues [45]. Studies have suggested that exposure to certain metals can inhibit MEG3 expression, such as chronic low-dose cadmium exposure in human bronchial epithelial cells and in nickel- and chromium-transformed cells [10, 18, 46]. In this study, we demonstrated that the downregulation of MEG3 in the arsenic-transformed human lung epithelial cells is caused by hypermethylation of its promoter. A previous study has shown that DNMT1 is involved in MEG3 downregulation in cells exposed to nickel and in bone marrow mesenchymal stem cells from the pediatric aplastic anemia [47]. In this study, DNMT3α, another member of the DNA methyltransferase family, is implicated in MEG3 repression. While DNMT3α/β are typically involved in de novo methylation during embryogenesis and germ cell development, their incorporation in promoter methylation during cancer sheds light on their novel function in cancer.

Given that cancer cells and malignant cells frequently displayed low MEG3 levels, we hypothesized that the downregulation of MEG3 may contribute to the acquired characteristics during malignant transformation induced by arsenic. To test the hypothesis, MEG3 was ectopically expressed in the As-T cells. We observed that cell migration and invasion potencies were decreased when the MEG3 expression levels were elevated, indicating that MEG3 is a negative regulator of cell migration and invasion in arsenic-transformed cells. Cancer development consists of three crucial steps: initiation, promotion, and progression. Cell migration and invasion represent the ability to migrate to a secondary site from the original site and to invade into vessels to spread through the body by the blood circulatory system. The last two processes of cancer development are largely dependent on migratory and invasive potencies. Previous studies revealed several pathways involved in the enhanced migration and invasion that were induced by arsenic exposure, including the activation of Akt and NRF2-mediated upregulation of SOX9 [48, 49]. Although NRF2 is an established regulator of NQO1 and is also involved in metastasis, the present study demonstrated that the negative regulation of MEG3 on NQO1 is independent of NRF2 (Supplementary Figure 3). The present study showed that MEG3 was lost in the As-T cells, followed by the upregulation of NQO1. It has been reported that NRF2/NQO1 promotes proliferation, cell migration, and colony formation in arsenic-transformed T-HaCaT cells through CDK2/Cyclin E [50]. The previous studies indicated that arsenic exposure downregulated NQO1 either through NRF2 or DNA methylation of the NQO1 promoter [50, 51]. Here, we have shown the presence of a novel intermediator MEG3 between arsenic exposure and NQO1 to regulate cell migration and invasion. Superoxide dismutase 2 (SOD2), known as manganese-dependent superoxide dismutase (MnSOD), mainly exists in mitochondria. This protein binds to the superoxide byproducts of oxidative phosphorylation and converts them to hydrogen peroxide and diatomic oxygen, playing the same role by different mechanisms as NQO1 in clearing mitochondrial reactive oxygen species (ROS). SOD2 was observed to be increased after MEG3 was knocked down and to be decreased after MEG3 was overexpressed in BEAS-2B cells (**Figure 3C**). SOD2 is not the focus of this study, however, the results demonstrated that loss of MEG3 did enhance the level

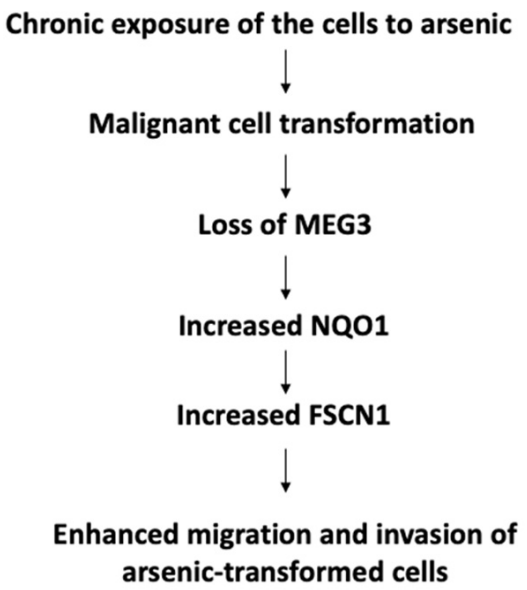

Figure 8. Mechanisms of increased migration and invasion in arsenic-induced carcinogenesis. Chronic exposure to arsenic induces cell malignant transformation, accompanied by the loss of MEG3. The downregulation of MEG3 upregulates the NQO1, which subsequently stabilized FSCN1 by the direct binding between NQO1 and FSCN1, leading to increased migration and invasion in arsenic-transformed cells.

of oxidants, to respond to which cells needed to upregulate NQO1 and SOD2. Further study is needed to investigate the mechanism by which loss of MEG3 increases oxidative stress and how cells respond to this change.

Previous studies have shown that NQO1 and FSCN1 are upregulated in human nasopharyngeal carcinoma based on a comprehensive analysis of the gene expression [52]. However, there have been no investigations into the relationship between these two genes. The present study revealed that NQO1 and FSCN1 proteins are downstream targets of MEG3, and FSCN1 is a direct downstream target of NQO1 as evidenced by (a) knockdown of NQO1 by its shRNA in arsenic-transformed cells decreased FSCN1 protein levels, but not mRNA level; (b) overexpression of NQO1 in normal BEAS-2B cells increased FSCN1 protein levels; (c) direct binding between these two proteins were detected. For the first time, we reported that NQO1 upregulates FSCN1 via their direct binding. Whereas NQO1 is also reported to stabilize HIF-1α as a protein chaperone, the expression level of HIF-1α is likely to be modulated by the level of NQO1 possibly through the interaction between NQO1 and HIF-1α oxygen-dependent degradation (ODD) domain. This interaction prevents HIF-1α from ubiquitination and then facilitates the nuclear translocation and the transcriptional factor role of HIF-1α [53]. As it has been reported, HIF-1α is a dominant transcription factor of FSCN1 by specifically targeting the hypoxia response element (HRE) of the FSCN1 promoter [54]. Hence, the regulation of NQO1 on FSCN1 could be partially due to stabilization through direct binding between NQO1 and FSCN1, or enhanced transcription of the FSCN1 gene induced by elevated HIF-1α. FSCN1 is one of the skeleton proteins which is involved in the actin protein package. The effect of FSCN1 on cell motility has been widely studied and accepted. In the present study, we observed a positive correlation between cell migration and invasion and the elevated FSCN1 protein level. Many studies have indicated that FSCN1 is regulated by microRNAs (miRs), such as miR-145, miR-133a/b, and miR-200b [32, 33, 55]. Recently, our laboratory has revealed the loss of MEG3 and upregulation of miR-145 play an important role in the invasion and migration of chromium (VI)-transformed cells [18]. The study conducted has revealed a previously undiscovered mechanism by which NQO1 regulates FSCN1 through direct binding. Analysis of expression using the TCGA database indicated a high expression of NQO1 and FSCN1 in all stages of human lung squamous cell carcinoma when compared to normal lung tissue. Our findings suggest that NQO1 and FSCN1 are two essential oncogenes that drive the progression of lung squamous cell carcinoma. We anticipate that this study serves as a foundation for future research and encourages the development of small-molecule drugs that target NQO1 or FSCN1.

In summary, this study revealed that the loss of MEG3 and increased expression of NQO1 and FSCN1 were associated with enhanced migration and invasion in arsenic-transformed cells. Overexpression of MEG3 decreased NQO1, causing suppression of the migration and invasion of arsenic-transformed cells. Furthermore, the re-expression of FSCN1 in BEAS-2B cells with stable NQO1 knockdown was found to reverse the inhibitory effect on migration and invasion. Collectively, our findings suggest that DNMT3α-mediated promoter hypermethylation of MEG3 results in the downregulation of MEG3, which in turn upregulates NQO1. NQO1 directly

binds to FSCN1, ultimately leading to enhanced migration and invasion of arsenic-transformed cells. The schematic representation of the proposed mechanisms of MEG3 on migration and invasion in arsenic-transformed cells is depicted in **Figure 8**.

Disclosure of conflict of interest

None.

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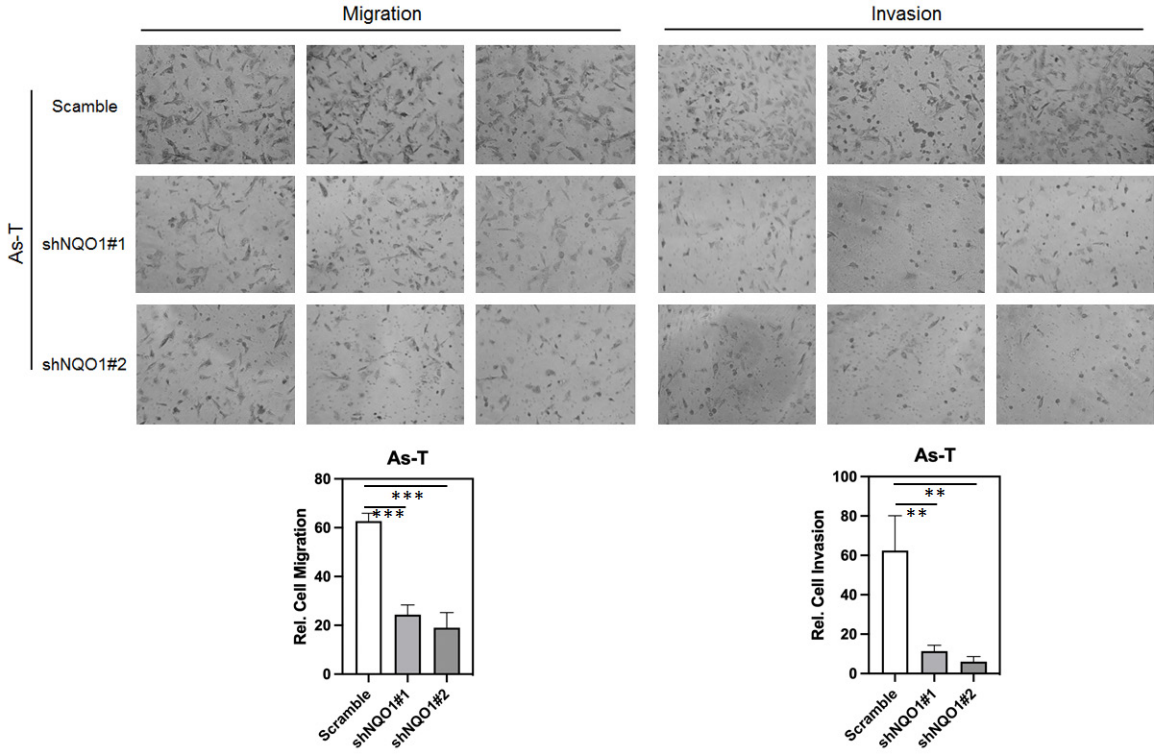
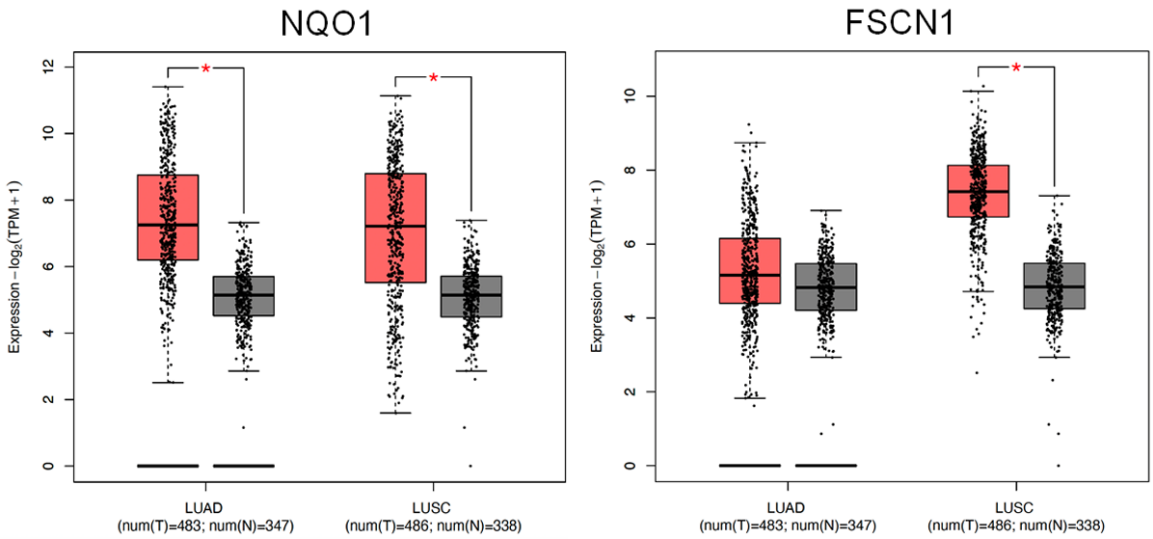
Supplementary Table 1. Lung Tissue Resource Involved in the Study

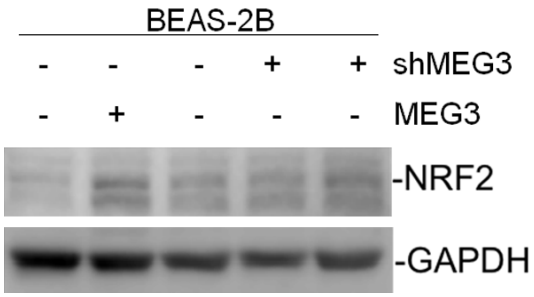
Code	Source	Age	Gender	Ethnicity	Smoking Status	Cancer Stage/Clinical Staging	Lymph Node Metastasis/ Pathologic Staging
SCC 1	Surgical resection	90	Male	White	Former; 30 years; quit in 1980s	Stage 1	pT1cN0
SCC 2	Surgical resection	70	Male	Not listed	Never smoked	Stage 2	pT3N0
SCC 3	Surgical resection	78	Female	White; Not of Spanish/ Hispanic Origin	Former; 60 pack year smoker, quit in 2006	Stage 3	pT2aN2
Control	Surgical resection	74	Female	White; Not of Spanish/ Hispanic Origin	Former; 20 pack-years, quit in 1989	Normal	Normal



Supplementary Figure 1. Knockdown of NQO1 abolished migration and invasion of As-T cells. Migration and invasion in As-T cells with or without NQO1 knockdown were evaluated by the transwell assays, images represent one of three images captured in each group. Migrated and invaded cells were quantified. ** and ***, $p<0.01$ and $p<0.001$, respectively.



Supplementary Figure 2. NQO1 and FSCN1 expression levels in human lung adenocarcinoma (LUAD) and human lung squamous cell carcinoma (LUSC) patients and normal people. NQO1 and FSCN1 expression levels from human lung adenocarcinoma (LUAD) and human lung squamous cell carcinoma (LUSC) tissues were analyzed and compared to the normal tissues using the TCGA database. *, $p<0.05$.



Supplementary Figure 3. MEG3 does not regulate NRF2 in BEAS-2B cells. Whole-cell lysates from BEAS-2B cells with or without MEG3 overexpression or knockdown were isolated for immunoblotting analysis.