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Inhibition of transforming growth factor beta/SMAD signal by MiR-155 is involved in arsenic trioxide-induced anti-angiogenesis in prostate cancer

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Key words

Angiogenesis, arsenic trioxide, DNA-demethylation, microRNA-155, TGF-β/SMAD signaling

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Prostate cancer is the most common cause of cancer-related deaths in men. Current practices for treatment of prostate cancer are less than satisfactory because of metastasis and recurrence, which are primarily attributed to angiogenesis. Hence, anti-angiogenesis treatment is becoming a promising new approach for prostate cancer therapy. In addition to treating acute promyelocytic leukemia, arsenic trioxide (As_2O_3) suppresses other solid tumors, including prostate cancer. However, the effects of As_2O_3 on angiogenesis in prostate cancer cells, and the underlying molecular mechanisms remain unclear. In the present study, As_2O_3 attenuated angiogenic ability through microRNA-155 (miR-155)-mediated inhibition of transforming growth factor beta (TGF-β)/SMAD signal pathway in human prostate cancer PC-3 and LNCaP cells *in vitro* and *in vivo*. Briefly, As_2O_3 inhibited the activations/expressions of both TGFβ-induced and endogenous SMAD2/3. Furthermore, As_2O_3 improved the expression of miR-155 via DNA-demethylation. MiR-155, which targeted the *SMAD2*-3′UTR, decreased the expression and function of SMAD2. Knockdown of miR-155 abolished the As_2O_3-induced inhibitions of the TGF-β/SMAD2 signaling, the vascular endothelial growth factor secretion and angiogenesis. Through understanding a novel mechanism whereby As_2O_3 inhibits angiogenic potential of prostate cancer cells, our study would help in the development of As_2O_3 as a potential chemopreventive agent when used alone or in combination with other current anticancer drugs.

Prostate cancer continues to represent a burgeoning medical problem in men.[1] In prostate cancer, most deaths are attributed to the metastatic diseases rather than the primary carcinomas.[1] In solid tumors, cancer cells recruit new blood vessels for their growth, maintenance and metastasis.[2] In fact, angiogenesis plays a crucial role in prostate cancer progression.[2] Studies have shown that vascular endothelial growth factor (VEGF) produced by prostate carcinoma cells functions as a growth factor and an angiogenesis inducer, which promotes tumor cells proliferation, migration and invasion.[3] Therefore, suppression of VEGF synthesis and/or inhibition of VEGF activity, which attenuates the tumor-induced development of new blood vessels, is an important strategy for prostate cancer treatment.

Arsenic trioxide (As_2O_3) has been used clinically as an anti-tumor agent, and can induce complete remission in patients with acute promyelocytic leukemia.[4] In addition, the anti-cancer effects of As_2O_3 have been observed in other solid tumors, such as liver, breast and prostate.[5–7] In most cases, inhibition of proliferation and/or induction of apoptosis by As_2O_3 in cancer cells is well characterized.[6] Recent studies reveal the novel functions of As_2O_3 in cancer therapy, that it attenuates the invasion potential of human liver cancer cells.[8] Furthermore, as a model agent, As_2O_3 causes significant vascular shutdown in brain, mammary and gastric cancers.[9,10] However, the molecular mechanisms underlying the effects of As_2O_3 on angiogenesis in prostate cancer remain unclear.

In a wide range of biological systems, signaling by the transforming growth factor beta (TGF-β) superfamily is involved in the regulation of cell growth, differentiation and development.[11] In cancer cells, TGF-β has conflicting effects during tumor progression, initially as a suppressor and then as a promoter.[11] In the progression of cancers, TGF-β induces the epithelial–mesenchymal transition (EMT), resulting in enhanced metastatic ability.[12] In prostate cancer, TGF-β signaling cascade has been implicated in the spread of prostate cancer since elevated serum levels of TGF-β were observed in patients with lymph node and distant site metastases compared to those with localized cancer.[13] Previous study indicates that blockage of TGF-β signaling significantly attenuates the VEGF secretion in prostate carcinoma cells.[2] Here we treated PC-3 and LNCaP cells with As_2O_3 to determine the early molecular changes, with emphasis on angiogenesis and TGF-β signaling.

Materials and Methods

Cell culture and reagents. Human prostate cancer PC-3 and LNCaP cells were obtained from the Shanghai Institute of Cell Biology (Shanghai, China). Cells were maintained in 5% CO_2 at 37°C in RPMI-1640 medium (Life Technologies/Gibco, Grand Island, NY, USA) containing 10% FBS (Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco). Arsenic trioxide (As_2O_3, 99.0% purity) was purchased from Sigma Chemical (St. Louis, MO, USA). All the other reagents used were of analytical grade or the highest grade available.

Animals. This study was performed according to a protocol approved by the Nanjing Medical University Institutional Animal Care and Use Committee, and animals were treated humanely and with regard for alleviation of suffering. For xenograft studies, 1×10^7 PC-3 cells were injected s.c. into the right armpit of the mice (6 mice per group). Three weeks later, As_2O_3 (2 mg/kg·BW) was administered i.p. twice per week. Tumor volumes were measured weekly using the formula: $V = ½ (\text{width}^2 \times \text{length})$. After 8 weeks, the mice were killed, and the tumor tissues were removed for further investigation.

ELISA. To analyze VEGF secretion, PC-3 and LNCaP cells were seeded in 6-well plates for 24 h. These cells were then serum starved for 12 h and treated with 1640 medium supplemented with 2% FBS, in the presence or absence ofAs_2O_3 for 48 h. The conditioned media was collected, cleared by centrifugation and stored at −80°C. ELISA was performed using the human VEGF Quantikine kit (R&D Systems, Minneapolis, USA) according to company protocol. Recombinant human VEGF was used for calibration.

Tube formation assay. The HUVEC were trypsinized and seeded at 5×10^4 cells per well in a 48-well plate on Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) that had polymerized for 30 min at 37°C. Then, the cells were incubated in the conditioned media as described above for 6 h, respectively. Capillary morphogenesis was evaluated using an inverted microscope (Olympus, Tokyo, Japan).

Western blots. Cell lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore, Billerica, MA, USA); the immune complexes were detected by enhanced chemiluminescence (Cell Signaling Technology, Beverly, MA, USA). Antibodies used were SMAD2, p-SMAD2 (Ser 465/467), SMAD3 and p-SMAD3 (Ser 423/425; Cell Signaling Technology); VEGF (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and GAPDH (Sigma Chemical).

Quantitative RT-PCR. The primers used are listed in Table S1. The miRNA primers were synthesized by RiboBio (Guangzhou, China). Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. The qRT-PCR was performed using an ABI 7300 real-time PCR detection system (Applied Biosystems by Life Technologies, Grand Island, NY, USA). Fold changes in expression of each gene were calculated by a comparative threshold cycle (C_t) method using the formula $2^{-(\Delta\Delta Ct)}$.

Cell transfection. Anti-con and anti-miR-155 were synthesized by RiboBio. Cells were transiently transfected using the Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's protocol. Briefly, cells were seeded in 6-well plates at a density of 1×10^5 per well. After 48 h, these cells were transfected with 100 nM of anti-miR-155 or anti-con for 12 h. After transfection, cells were conventional cultured for another 24 h before being used for other experiments.

Immunohistochemistry and angiogenesis quantification. Sections mounted on silanized slides were dewaxed in xylene, dehydrated in ethanol, boiled in 0.01 M citrate buffer, pH 6.0, for 20 min in a microwave oven, and then incubated with 3% hydrogen peroxide for 5 min. After washing with PBS, sections were incubated in 10% normal bovine serum albumin for 5 min, followed by overnight incubation with a rabbitanti-human VEGF and CD31 (Santa Cruz Biotechnology) antibody, and then with an anti-rabbit horseradish peroxidase-conjugated secondary antibody (Beyotime) for 30 min. The sections were counterstained with hematoxylin, dehydrated, cleared and mounted. For tumor-associated angiogenesis quantification, microvessel density (MVD) was evaluated by counting CD31-positive immunostained cells, as described previously.[14] The average count of five vision fields was recorded as the final MVD.

DNA methylation analysis. Cellular DNA was isolated using DNA purification kits (Qiagen, Germantown, MD, USA). The genomic DNA was modified with sodium bisulfite using the EpiTect Kit (Qiagen). DNA methylation was analyzed by quantitative methylation-specific PCR (qMSP), as described previously.[15] The primers used are listed in Table S2. The percentage of methylation in a sample was estimated using the formula: $\text{Methylation } (\%) = (M/M + U) \times 100\% = [1/(1 + U/M)] \times 100\% = [1/(1 + 2^{\Delta Ct})] \times 100\%$. Annotation: M, methylated; U, unmethylated.

Statistical analysis. Data were presented as the means ± SD. Student's *t*-test, and one-way ANOVA followed by Dunnett's *t*-test were used to assess significant differences between groups. *P*-values < 0.05 were considered statistically significant.

Results

As_2O_3 attenuates angiogenic abilities in prostate carcinoma cells. First, we determined the effects of As_2O_3 on the viabilities of human prostate carcinoma PC-3 and LNCaP cells. As shown in Figure 1(a), there were decreases of viabilities in cells exposed to 4 μM As_2O_3; however, no detectable attenuations of viabilities were observed in cells exposed to 0.5, 1 and 2 μM As_2O_3. Next, we used ELISA to investigate the effects of As_2O_3 on the VEGF secretion in these cells. As shown in Figure 1(b), As_2O_3 inhibited the secretions of VEGF in a dose-dependent manner. Finally, we used the tube formation assay to further detect the functions of As_2O_3 in the angiogenic ability of prostate carcinoma cells. To avoid the addition of As_2O_3 to the endothelial cells in the tube formation assay, we pre-treated PC-3 and/or LNCaP cells with 0 or 2 μM As_2O_3 for 48 h, followed by a conventional culture in fresh medium for another 24 h. The conditioned mediums were then collected. We treated human umbilical vein endothelial cells (HUVEC) with these conditioned mediums, respectively. Our data showed that there was decreased cell viability (Fig. S1a) and attenuated formations of tubes in HUVEC (Fig. 1c,d) incubated with the conditioned mediums collected from As_2O_3-pre-treated cells. Importantly, there was no detectable inhibition of tube formation after HUVEC were directly exposed to As_2O_3 (Fig. S1b). These results suggest that As_2O_3 attenuates the angiogenic abilities of prostate carcinoma cells, in which the inhibitions of VEGF secretion are involved.

As_2O_3 blocks the transforming growth factor beta/SMAD signaling. The TGF-β/SMAD signal pathway plays an important role in the VEGF secretion in human prostate carcinoma cells.[2] We first examined the effects of As_2O_3 on the activation of SMAD2 and SMAD3 in TGF-β1-treated PC-3 cells. As shown in Figure 2(a), As_2O_3 blocked the TGF-β1-induced ele-

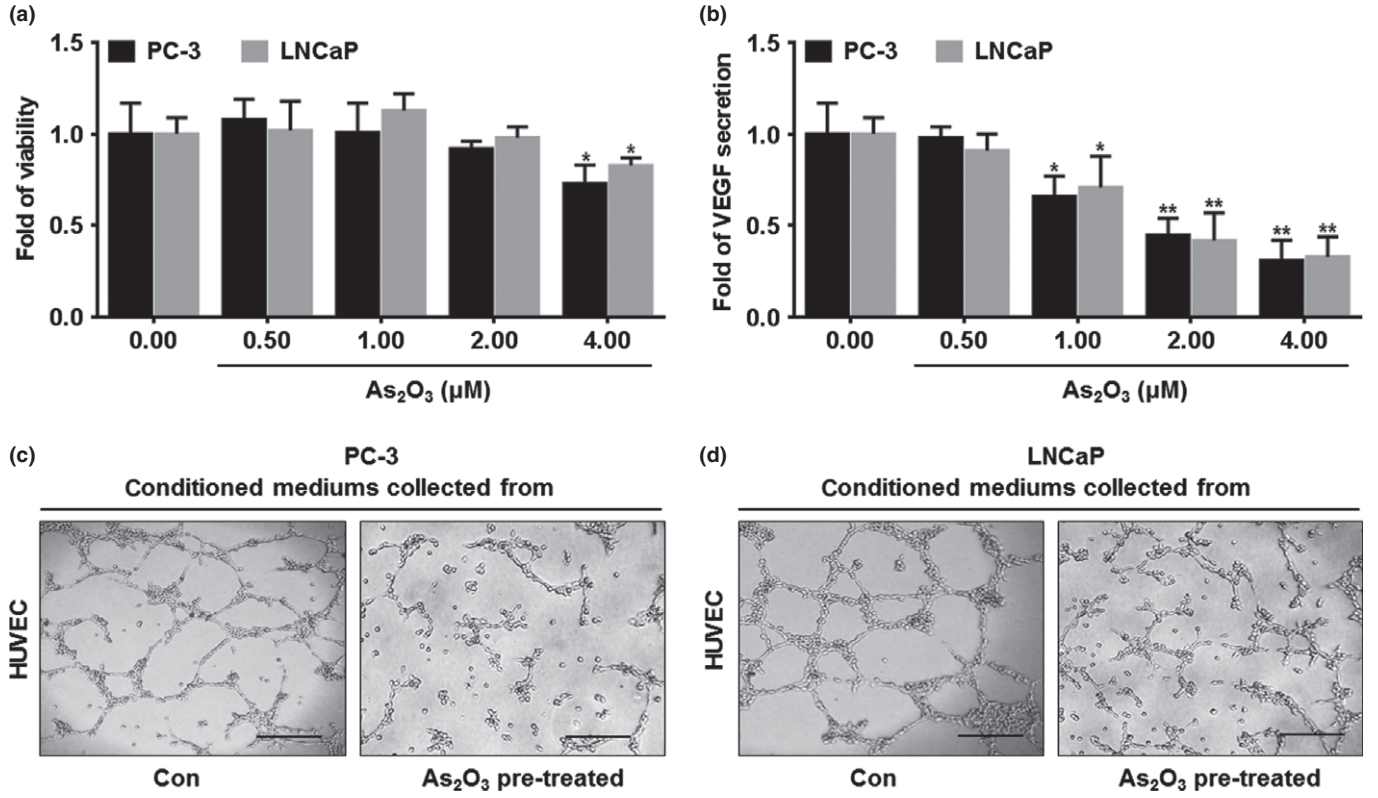

Fig. 1. As_2O_3 attenuates the angiogenic abilities in prostate carcinoma cells. (a and b) PC-3 and LNCaP cells were treated by 0, 0.5, 1, 2, or 4 μM As_2O_3 for 48 h, respectively, and the mediums were collected for further investigation. (a) The cell viabilities were evaluated in triplicate by WST-8 hydrolysis using a Cell Counting Kit-8 assay. (b) The vascular endothelial growth factor (VEGF) secreted by PC-3 and/or LNCaP cells in the mediums were determined in triplicate by ELISA. *$P < 0.05$ and **$P < 0.01$ compared with medium control cells.(c and d) After PC-3 (c) or LNCaP (d) cells were pre-treated by 0 or 2 μM As_2O_3 for 48 h, followed by a conventional culture in fresh medium for another 24 h, the mediums were collected. Human umbilical vein endothelial cells (HUVEC) were exposed to such mediums as indicated for 6 h, respectively, and the formations of tube were evaluated as described in the section "Materials and Methods". Bars = 250 μm.

vation of p-SMAD2, p-SMAD3 and VEGF (a downstream factor regulated by TGF-β[16,17]) in a dose-dependent manner. Moreover, As_2O_3 also attenuated the TGF-β1-induced increased expression of *VEGF* mRNA in PC-3 and LNCaP cells (Fig. 2b). Next, we determined the effects of As_2O_3 on the expressions/activations of endogenous SMAD2 and SMAD3 in prostate carcinoma cells. Our data showed that either As_2O_3 or SB431542 (TGF-β type I receptor inhibitor) could decrease the activations of endogenous TGFβ/SMAD signal (Fig. S2, as determined by the phosphorylation of SMAD2 and the expression/secretion of VEGF) and (Fig. 2c–e, as determined by phosphorylation of SMAD2/3 and the expressions of PAI-1 and SMAD7, two faithful target genes of TGF-β signal). Moreover, As_2O_3 also decreased the expressions of total SMAD2 and SMAD3 proteins and mRNA (Fig. 2c,f,g). These results indicate that As_2O_3 inhibits the TGF-β-SMAD signal pathway in PC-3 and LNCaP cells, and that the repressive effects of As_2O_3 on the endogenous SMAD2/3 may be mediated by the microRNA (miRNA).

As_2O_3 improves the miRNA-155 expression by DNA demethylation. Using TargetScan 6.2 (www.targetscan.org) we found that miR-155 was predicted to bind the *SMAD2*-3′UTR. The target sites of miR-155 in *SMAD2* mRNA are exhibited in Figure S3. Previous study also indicates that miR-155 targets SMAD2 and modulates the response of macrophages to TGF-β.[18] Therefore, we first determined the effects of As_2O_3 on the expression of miR-155 in prostate carcinoma cells. As shown in Figure 3(a), As_2O_3 improved the expression of miR-155 in a time-dependent manner in PC-3 and LNCaP cells. Next, we investigated the molecular mechanisms whereby As_2O_3 upregulated miR-155. It has been reported that miR-155 is epigenetically silenced in human cancers.[19,20] Interestingly, biotransformation of arsenic results in a deficiency of methyl donors, reducing DNA methylation, which activates a series of target genes (Fig. 3b).[21] Hence, we hypothesized that As_2O_3 elevates the expression of miR-155 by DNA demethylation. To confirm this hypothesis, qMSP was conducted. As shown in Figure 3(c), As_2O_3 decreased the average methylation level of miR-155 promoter in PC-3 cells. Then, we further tested the functional relevance of As_2O_3-induced DNA demethylation and increased expression of miR-155. As shown in Figure 3(d), hypermethylation treatment by SAM dramatically blocked the As_2O_3-induced elevation of miR-155. These results suggest thatAs_2O_3 upregulates the expression of miR-155 by demethylation in PC-3 and LNCaP cells.

MiR-155 is involved in the As_2O_3-induced inhibition of transforming growth factor beta/SMAD2 and angiogenic abilities in prostate carcinoma cells. Based on the factor that miR-155 targets *SMAD2* mRNA[18] and that As_2O_3 elevates the expression of miR-155, we hypothesized that miR-155 might be involved in the As_2O_3-induced decreased expression/activation of SMAD2. Here, knockdown of miR-155 (Fig. 4a) blocked the As_2O_3-induced inhibition of SMAD2 in PC-3 and LNCaP cells, as determined by the decreased expressions of SMAD2, p-SMAD2 and VEGF (Fig. 4b–d). Then, we determined the functions of miR-155 in the As_2O_3-induced inhibition of

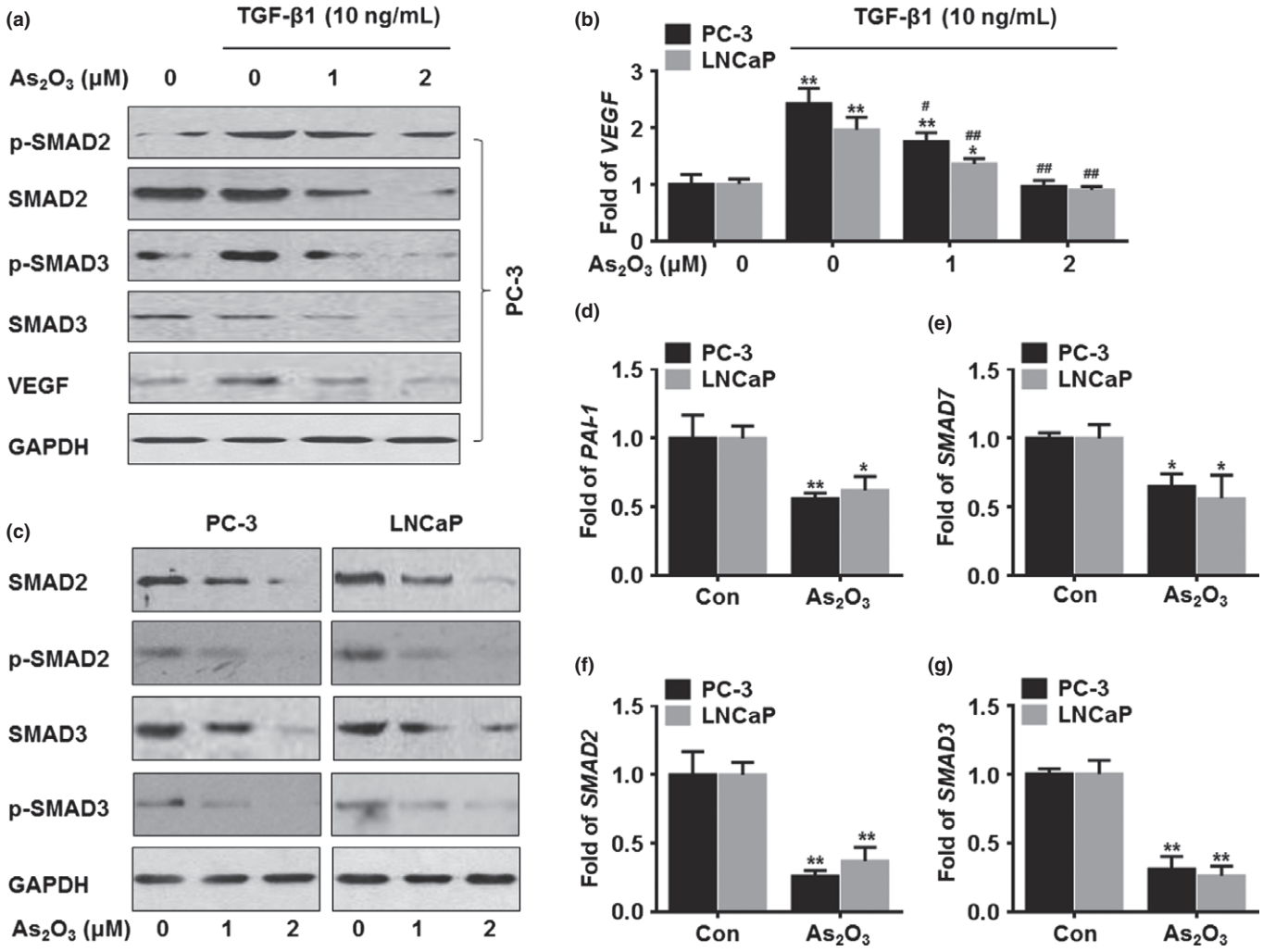

Fig. 2. As_2O_3blocks the transforming growth factor beta (TGF-β)/SMAD signaling. (a and b) After PC-3 cells were pre-treated by 0, 1 or 2 μM As_2O_3 for 24 h, they were exposed to 0 or 10 ng/mL TGF-β1 for 24 h. (a) Western blots analyses of the p-SMAD2, SMAD2, p-SMAD3, SMAD3 and vascular endothelial growth factor (VEGF) proteins. (b) Quantitative (qRT-PCR) analyses in triplicate of the *VEGF* mRNA. (c–g) PC-3 and LNCaP cells were treated by 0, 1 or 2 μM As_2O_3 for 24 h, respectively; (c) Western blots analyses of the SMAD2, p-SMAD2, SMAD3 and p-SMAD3 proteins. qRT-PCR analysesin triplicate of the (d) *PAI-1*, (e) *SMAD7*, (f) *SMAD2* and (g) *SMAD3* mRNA.$^{**}P < 0.01$ compared with medium control cells; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with cells treated by TGF-β1alone.

angiogenic abilities in prostate carcinoma cells. After PC-3 and/or LNCaP cells were pre-transfected by anti-con or anti-miR-155 for 12 h, they were exposed to 0 or 2 μM As_2O_3 for 48 h, followed by a conventional culture in fresh medium for another 24 h. The conditioned mediums were then collected. As shown in Figure 5(a), knockdown of miR-155 blocked the As_2O_3-induced inhibitions of VEGF secretion. Furthermore, there was decreased cell viability and attenuated formations of tubes in HUVEC incubated with the conditioned mediums collected from As_2O_3-pre-treated cells; however, knockdown of miR-155 abolished this phenomenon (Fig. 5b,c). These results indicated that As_2O_3 blocked the TGF-β/SMAD2 signal and the angiogenic abilities of prostate cancer cells by miR-155.

Effects of As_2O_3 on miR-155/SMAD2 and on angiogenesis *in vivo*. Finally, we investigated the effects of As_2O_3 on the miR-155/SMAD2 signal pathway and on angiogenesis *in vivo*. As shown in Figure 6(a), As_2O_3 decreased the tumor growth. However, the body weight and liver weight were similar in both vehicle and CaA-treated mice (Table S3). In addition, in the As_2O_3-treated group, there was increased expression of miR-155 but decreased expressions of *VEGF* and *SMAD2* mRNA (Fig. 6b–d). Importantly, As_2O_3 exposure decreased the expressions of CD31 (a vessel density marker[22]) mRNA and protein (Fig. 6e,f). The MVD was also decreased by As_2O_3 (Fig. 6g). These results indicate that As_2O_3 improved the miR-155 but inhibited the SMAD2 and angiogenesis *in vivo*.

Discussion

Inorganic arsenic functions as a double-edged sword to human health. Although epidemiologic evidence implicates exposure to arsenic in causing human cancers of the skin, lung and bladder, arsenic has been used clinically as an anti-tumor agent in leukemia as well as other solid tumors.[23,24] Studies reveal that As_2O_3 affects the tumor/cell growth through a complicated process, including the regulation of signal pathways, transcriptional factors and microRNA.[5,8] In human cells, the proliferation is also regulated by a variety of complicated progressions, which control the cell cycle.[25,26] Furthermore, when tumor cells suffer form DNA damages, the compensations of cell growth/proliferation are initiated.[27] Hence, the

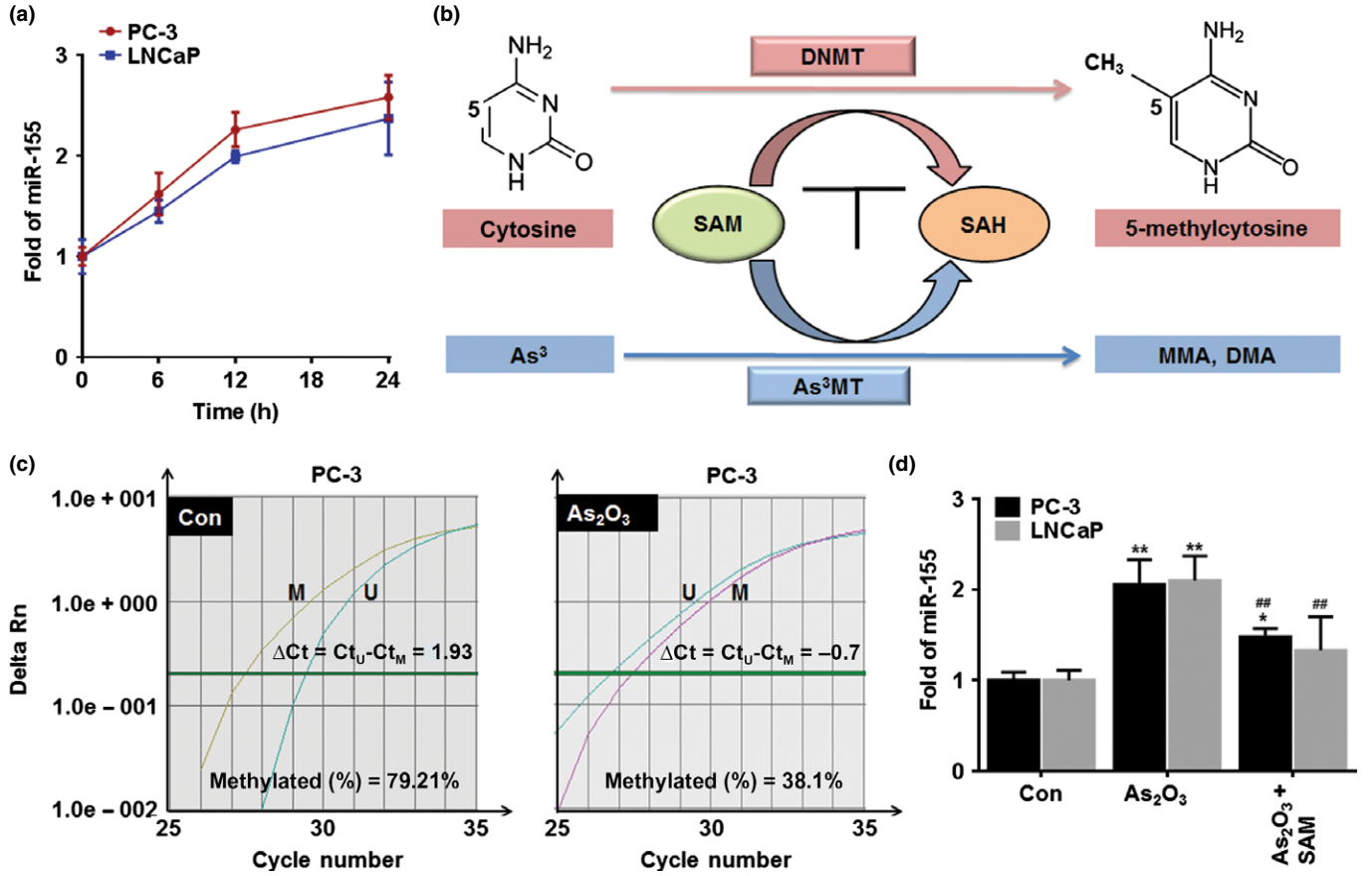

Fig. 3. As_2O_3 improves the miR-155 expression by DNA demethylation. (a) PC-3 and LNCaP cells were treated by 0 or 2 μM As_2O_3 for 6, 12 or 24 h, respectively; quantitative (qRT-PCR) analyses in triplicate of the miR-155. (b) Schematic illustration of the enzymatic DNA methylation and itsmodulation by the As^{3+}MT-mediated metabolic methylation of As_2O_3. During the As^{3+}MT-mediated metabolic methylation of As_2O_3, there was a deficiency of methyl donors (SAM); however, the formation of SAH would be markedly increased. It is hypothesized that elevated intracellular levels of SAH due to metabolic methylation of As_2O_3 mayexert a strong feedback inhibition of DNMT-mediated DNA methylation. (c) PC-3 cells were treated by 0 or 2 μM As_2O_3 for 24 h, and the methylation status of miR-155 promoter region was determined by qMSP. M, methylated; U, unmethylated. (d) PC-3 and LNCaP cells were treated by 0 or 2 μM As_2O_3 in the presence or absence of 200 μM SAM for 24 h, respectively; qRT-PCR analysis in triplicate of the miR-155. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with medium control cells; $^{\#\#}P < 0.01$ compared with cells treated by As_2O_3 alone.

molecular mechanisms underlying in the repressive effects of arsenic on angiogenesis in prostate cancer are very complicated. The present study indicates one of the potential mechanisms, that the miR-155-mediated inhibition of SMAD2 is involved in the As_2O_3-induced attenuation of angiogenesis in prostate cancer cells. Our results are in line with older data showing that apigenin (an anti-tumor agent) inhibits VEGF secretion by blocking the TGF-β/SMAD signal in prostate cancer cells; however, no detectable cytotoxicity is observed.[2]

In general, the TGF-β signaling is initiated with ligand-induced oligomerization of serine/threonine receptor kinases and phosphorylation of the cytoplasmic signaling molecules, Smad2 and Smad3.[28] Although the switch of TGF-β from a tumor suppressor to a tumor promoter is not well-defined, more evidence suggests that TGF-β plays an important role as the activator of the tumor growth and metastasis. Therefore, the TGF-β inhibitors are being developed as anti-metastatic therapy for patients with cancer.[26,29] For angiogenesis, two major mechanisms that regulate the VEGF secretionin prostate cancer cells have been identified, the hypoxia-regulated signaling pathways and the TGF-βsignaling pathways, both of which synergize in the regulation of *VEGF* gene expression at the transcriptional level.[30] Indeed, there was a positive feedback loop between TGF-β signal and VEGF. On one hand, TGF-β upregulates the-*VEGF* gene expression;[2] on the other hand, VEGF stimulation induces TGF-β1 expression.[31] However, in human endothelial cells (HUVEC), specifically, VEGF attenuates the activation of TGF-β effectively.[32] Here, As_2O_3 had no direct effect on VEGF-induced tube formation in HUVEC cells. One possible reason is: the background activation of TGF/SMAD signaling in HUVEC is attenuated by VEGF. Hence, although there would be increased expression of miR-155 in HUVEC, the target (SMAD2) has already been inactivated.

MiRNA are small non-coding RNA that regulate gene expression by binding to the 3′-UTR of the target mRNA and inhibiting translation or targeting the mRNA for degradation.[18] MiR-155 is one the most studied miRNA and is a typical multifunctional miRNA.[33] On one hand, by targeting TP53INP1, VHL, PIK3R1 and so on, miR-155 could promote cell proliferation, inhibit apoptosis and enhance tumor growth in numerous cancer cell lines and xenograft models, indicating that it may serve as an oncogenefor therapeutic intervention.[33] On the other hand, miR-155 may also function as a tumor suppressor to regulate cancer cell proliferation, invasion and metastasis by targeting CLDN-1 or SMAD2.[33] However, the roles of miR-155 in prostate cancer and the underlying molecular mechanisms remain largely uninvestigated. Here in As_2O_3-treated PC-3 and LNCaP cells, we identified that

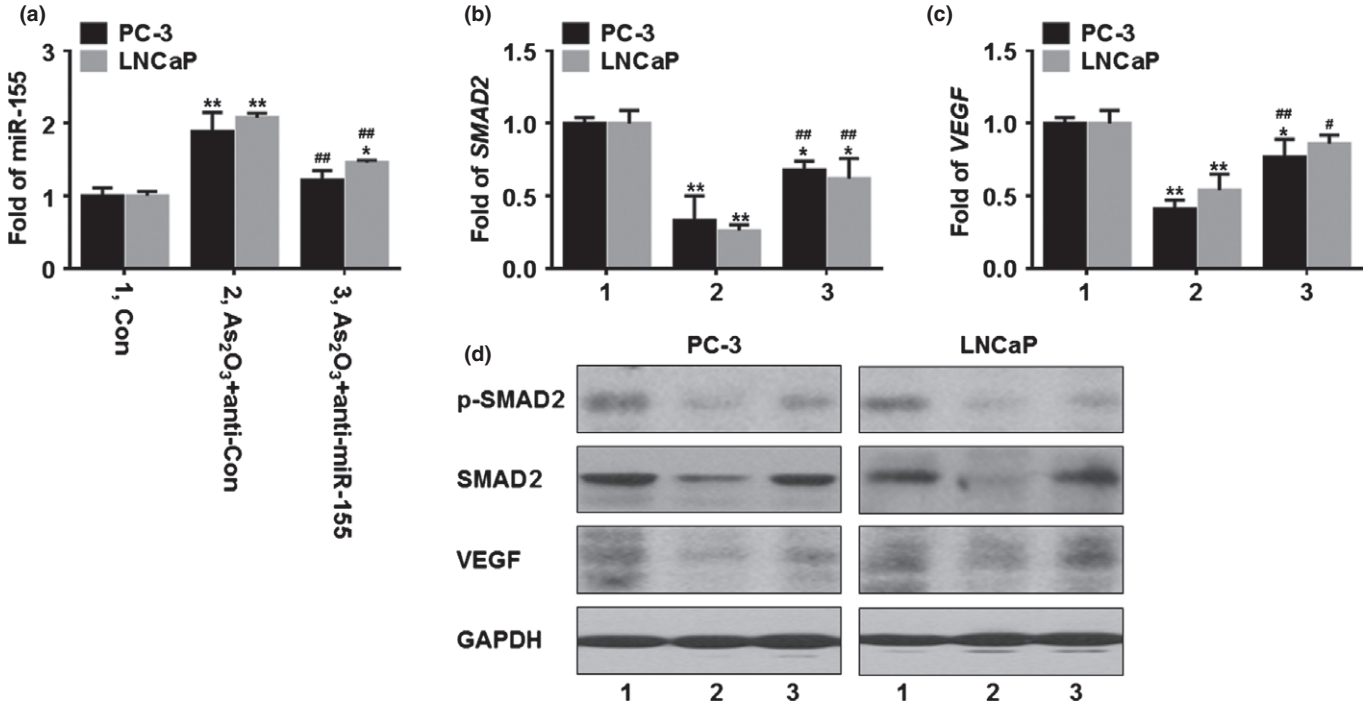

Fig. 4. MiR-155 is involved in the As_2O_3-induced inhibition of transforming growth factor beta (TGF-β)/SMAD2. After PC-3 and/or LNCaP cells were pre-transfected by anti-con or anti-miR-155 for 12 h, they were exposed to 0 or 2 μM $As_2O_3$for 24 h. Quantitative RT-PCR analysis in triplicate of the (a) miR-155, (b) *SMAD2* and (c) *VEGF* mRNA. (d) Western blots analyses of the p-SMAD2, SMAD2 and vascular endothelial growth factor (VEGF) proteins. $^{**}P < 0.01$ compared with medium control cells; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with cells transfected by anti-con.

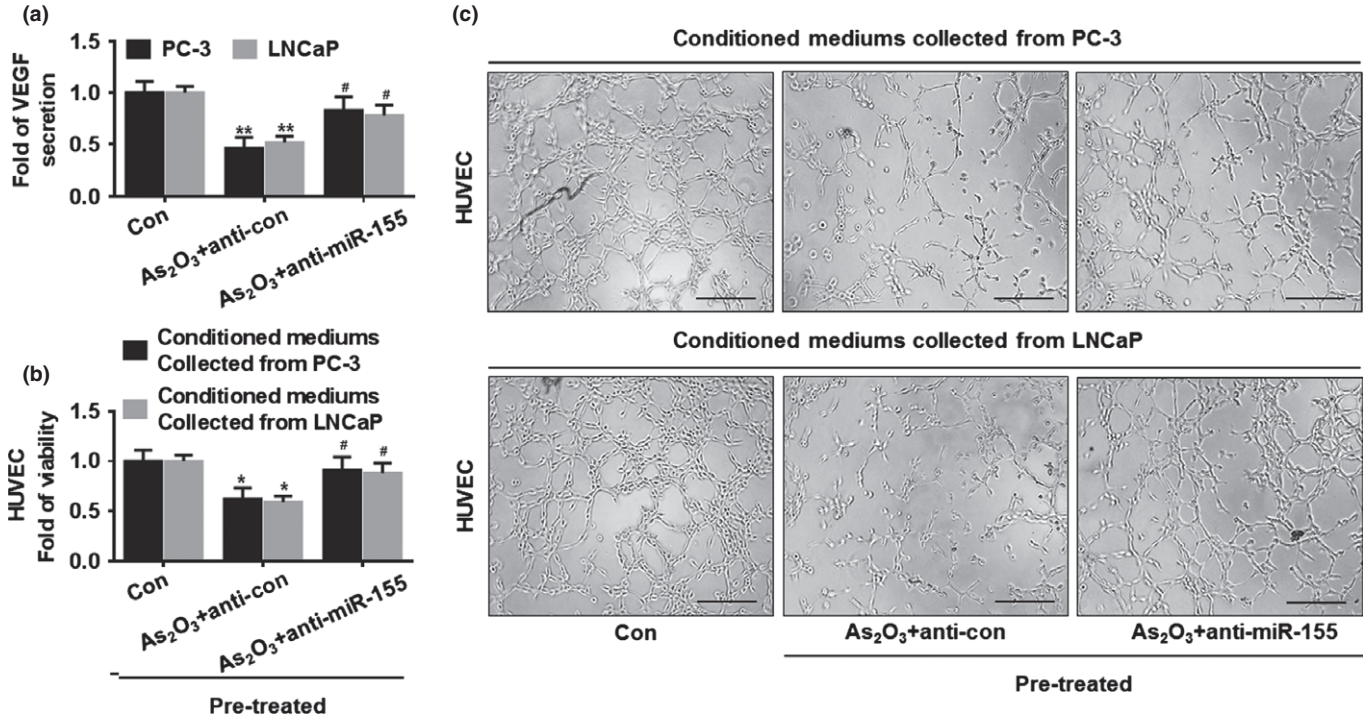

Fig. 5. As_2O_3 attenuates the angiogenic abilities by miR-155. After PC-3 and/or LNCaP cells were pre-transfected by anti-con or anti-miR-155 for 12 h, they were exposed to 0 or 2 μM As_2O_3 for 48 h, followed by a conventional culture in fresh medium for another 24 h. The conditioned mediums were then collected. (a) The vascular endothelial growth factor (VEGF) secreted by PC-3 and/or LNCaP cells in the mediums were determined in triplicate by ELISA. (b) The cell viabilities of human umbilical vein endothelial cells (HUVEC) cells were evaluated in triplicate by WST-8 hydrolysis using a Cell Counting Kit-8 assay. (c) The formations of tubes were evaluated as described in the section "Materials and Methods". Bars = 250 μm. $^{**}P < 0.01$ compared with medium control cells; $^{\#}P < 0.05$ compared with cells transfected by anti-con.

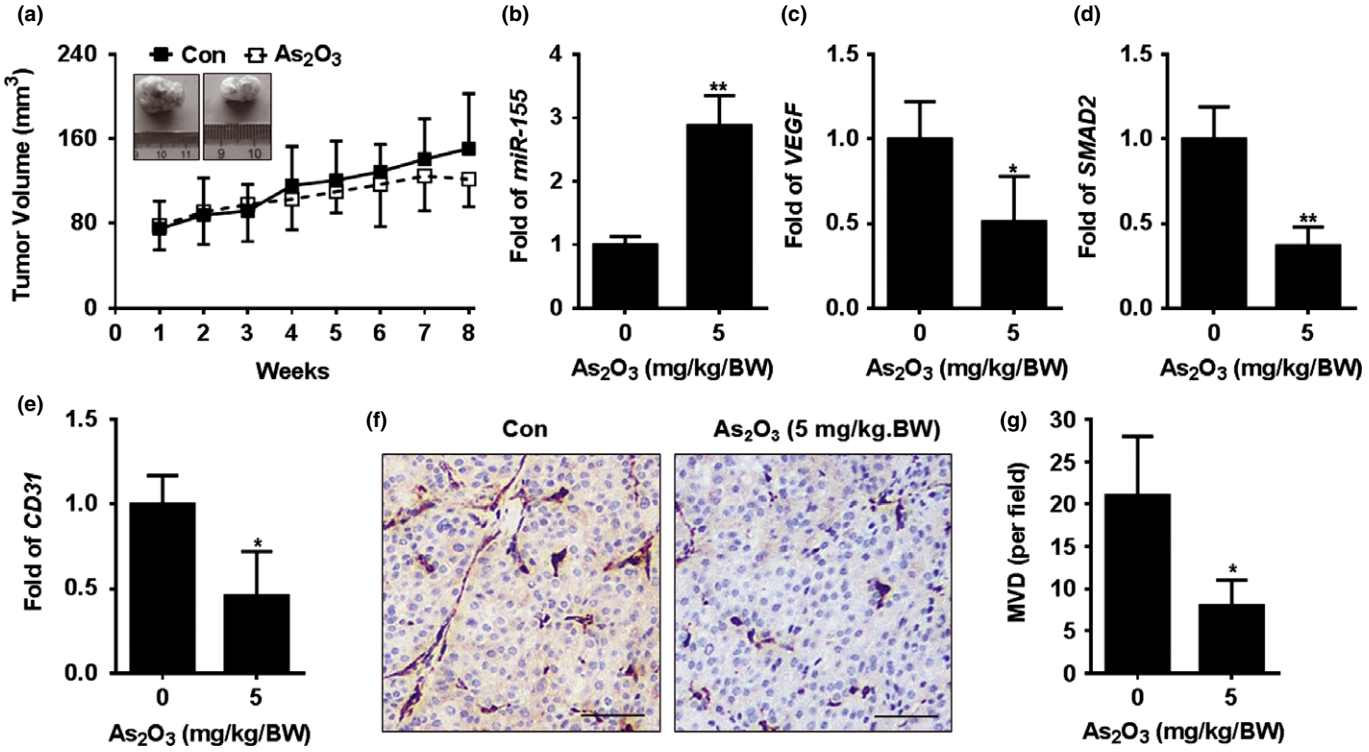

Fig. 6. Effects of As_2O_3 on miR-155/SMAD2 and on the angiogenesis *in vivo*. After PC-3 cells were injected s.c. into the right armpit of the mice for 3 weeks, As_2O_3 (0 or 2 mg/kg·BW) was administered (i.p.) twice per week. After 8 weeks, the mice were killed, and the tumor tissues were removed for further investigation. (a) Tumor images (top) and tumor volumes (bottom) were measured weekly and tumor size was calculated using the formula: $V = ½ (\text{width}^2 \times \text{length})$; (b–e) Total RNA isolated from tumors ($n = 6$) were mixed, quantitative RT-PCR analyses in triplicate of the (b) miR-155, (c) *VEGF*, (d) *SMAD2* and (e) *CD31*mRNAs. (f) Immunohistochemistry analyses of CD31 (bar = 100 μm). (g) Angiogenesis quantification by microvessel density. $^*P < 0.05$ and $^{**}P < 0.01$ compared with mice treated with no As_2O_3.

miR-155 exhibited a tumor suppressor function, which attenuated the VEGF secretion and angiogenesis by targeting SMAD2. Furthermore, our data also revealed that the elevation of miR-155 by As_2O_3 was dependent on the DNAdemethylation.

DNA-demethylation plays an important role in the antitumor effects induced by As_2O_3.[21] Methylation of arsenic requires SAM as a cofactor and, as yet, largely uncharacterized methyltransferases (MeTases), including DNA MeTases (DNMT), a group of enzymes responsible for DNA methyla-

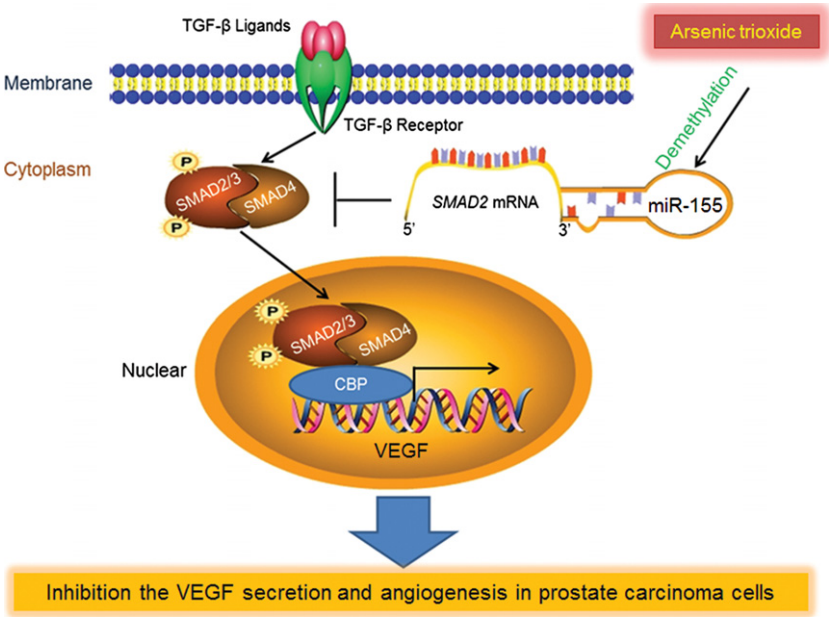

Fig. 7. Inhibition of transforming growth factor beta (TGF-β)/SMAD signal by demethylation-activated miR-155 is involved in arsenic trioxide-induced anti-angiogenesis in prostate cancer. VEGF, vascular endothelial growth factor.

tion.[21] As demonstrated in our previous study, As_2O_3 decreases the expressions of DNMT1, DNMT3a and DNMT3b, which epigenetically re-expressed the functional estrogen receptor α in triple negative breast cancer (TNBC) cells, and sensitizes these cells to tamoxifen therapy.[34] In addition, such a process is also involved in the upregulation of the miR-200 family and miR-491, which blocks the EMT and attenuates the migration and invasion potentials in TNBC and liver cancer cells.[8,35] Previous studies indicate that miR-155 is epigenetically silenced in human cancers.[19,20] Here, in the present study, the average methylation level of miR-155 promoter in PC-3 cells is 79.21%, while the average methylation level of miR-155 promoter in As_2O_3-treated PC-3 cells is 38.1%. Furthermore, hypermethylation treatment by SAM dramatically blocked the As_2O_3-induced elevation of miR-155.

Collectively, in human prostate cancer cells, As_2O_3 blocked angiogenesis through the inhibition of TGF-β/SMAD signal pathway *in vitro* and *in vivo*. Indeed, As_2O_3 improved the expression of miR-155 via DNA-demethylation. As an upstream regulator of TGF-β signaling, miR-155 decreased the expression and function of SMAD2 by targeting the *SMAD2*-3′UTR. Knockdown of miR-155 abolished the As_2O_3-induced inhibitions of TGF-β/SMAD2 signaling, VEGF secretion and angiogenesis (Fig. 7).

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Disclosure Statement

The authors have no conflict of interest to declare.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1. Primers used for quantitative RT-PCR.

Table S2. Primers used for quantitative methylation-specific PCR.

Table S3. Liver weight and body weight in nude mice treated with vehicle (physiological saline) or As2O3 (5 mg/Kg•BW).

Fig. S1. Effects of As_2O_3 on the tube formation in vascular endothelial growth factor (VEGF)-treated human umbilical vein endothelial cells (HUVEC).

Fig. S2. Effects of SB431542 on the vascular endothelial growth factor (VEGF) expression/secretion in PC-3 cells.

Fig. S3. The target sequences of miR-155 in the 3′-UTR of SMAD2 mRNA.

Fig. S4. Effects of As_2O_3-pretreated on the expression/activation of miR-155 and SMAD2.