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# Identification of Id1 as a downstream effector for arsenic-promoted angiogenesis via PI3K/Akt, NF-κB and NOS signaling

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## Abstract

Exposure to arsenic is known as a risk factor in various types of cancer. Apart from its carcinogenic activity, arsenic also shows promoting effects on angiogenesis, a crucial process for tumor growth. Yet, the mechanism underlying arsenic-induced angiogenesis is not fully understood. In this study, we aimed at investigating the involvement of inhibitor of DNA binding 1 (Id1) and associating signal molecules in the arsenic-mediated angiogenesis. Our initial screening revealed that treatment with low concentrations of arsenic (0.5–1 μM) led to multiple cellular responses, including the enhanced endothelial cell viability and angiogenic activity as well as the increased protein expression of Id1. The arsenic-induced angiogenesis was suppressed in the Id1-knockdowned cells compared with that in control cells. Furthermore, arsenic-induced Id1 expression and angiogenic activity were regulated by PI3K/Akt, NF-κB, and nitric oxide synthase (NOS) signaling. In summary, our current data demonstrate for the first time that Id1 mediates the arsenic-promoted angiogenesis, and Id1 may be regarded as an antiangiogenesis target for treatment of arsenic-associated cancer.

## Introduction

Arsenic (As) is a metalloid widely distributed in nature via its multiple forms in association with other elements.[1] Despite an ecological role, arsenic exposure has been shown pernicious to human health.[2] For instance, intake of arsenic-contaminated drinking water, food, or air causes renal and urinary dysfunction and elevated cancer incidence.[3-5] Exposure to arsenic during early gestation is also adversely correlated with long-term health issues, including increased incidence of carcinogenesis in later life.[6-8]

Angiogenesis is implicated in many pathological conditions such as rheumatoid arthritis,[9] wound healing,[10] cerebral ischemia,[11] and cardiovascular or peripheral artery diseases.[12, 13] In addition, tumorigenesis is largely dependent on neovascular formation, and therefore antiangiogenic therapy has become one of the main therapeutic strategies to control tumor growth.[14, 15] While most of the on-market antiangiogenic agents are targeting vascular endothelial growth factor (VEGF) and its downstream signaling cascade, clinical response to these agents is modest and new targeted therapies remain to be developed.[16]

Inhibitor of DNA binding 1 (Id1), also known as inhibitor of differentiation 1, is a member of basic helix-loop-helix (bHLH) transcription factor proteins.[17, 18] Structurally, Id1 is lack of the basic region that is adjacent to the HLH domain and essential for DNA binding.[17] Without a capacity of DNA binding by itself, Id1 forms heterodimers with other bHLH transcription factors which regulate the expression of genes involved in cell proliferation and differentiation.[18] Notably, upregulation of Id1 expression has been correlated with cancer progression, suggesting that Id1 may constitute an important target for anticancer therapeutics.[19-22]

It has been shown that arsenic exerts a dual effect on angiogenesis and carcinogenesis.[23-25] Intriguingly, Id1 also plays a regulatory role for tumor angiogenesis.[26-28] However, whether Id1 is involved in arsenic-associated angiogenesis has not been elucidated. In this study, we report that knockdown of Id1 expression compromises the arsenic-promoted angiogenesis. In addition, the arsenic-induced Id1 expression and angiogenesis are suppressed by inhibition of PI3K/Akt, NF-κB and nitric oxide synthase (NOS) signaling pathway. Together, our current results provide a novel finding for the involvement of Id1 in arsenic-associated angiogenesis.

## Materials and methods

### Materials

Sodium arsenite, wortmannin, QNZ, DETA-NONOate, 1400W dihydrochloride, L-NAME hydrochloride, basic FGF, and heparin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Matrigel was purchased from BD Biosciences (San Jose, CA, USA). Dulbecco's modified eagle medium (DMEM) was purchased from Gibco (Carlsbad, CA, USA). Fetal bovine serum, penicillin, streptomycin, and amphotericin B were purchased from Biological Industries (Beit-Haemek, Israel). Other reagents employed in this study were indicated separately wherever suitable.

### Cell culture

Mouse pancreatic endothelial cells (MS1) and human umbilical vein endothelial cells (HUVEC) were obtained from the American Type Culture Collection (Manassas, VA, USA). MS1 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. HUVEC cells were cultured in EndoGRO-LS complete medium (Millipore, MA, USA). All cells were maintained at 37°C in a 5% $CO_2$ incubator.

## Cell viability assay

MS1 or HUVEC cells were seeded at a density of $3\times10^3$ cells/well in 96-well plates for 24 h prior to treatment with the indicated concentrations of arsenic. After 72 h of treatment, the number of viable cells was determined by XTT assay (Sigma-Aldrich) according to the manufacturer's instructions.

## Western blot

Protein extracts of cells were prepared in lysis buffer (50 mM Tris-HCl pH 7.6, 120 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM β-mercaptoethanol, 50 mM NaF, and 1 mM $Na_3VO_4$), followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting analysis as described previously.[29] Antibody against Id1 was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies against STAT3, Akt, phospho-Akt (Ser473), p65, phospho-p65 (Ser536), iNOS, and β-actin were from GeneTex (Irvine, CA, USA). Antibodies against phospho-STAT3 (Tyr705), phospho-STAT3 (Ser727), phosphor-eNOS (Ser1177), and eNOS were from Cell Signaling Technology (Danvers, MA, USA).

## In vitro angiogenic tube formation assay

The *in vitro* angiogenic tube formation was carried out according to a previous report.[30] Briefly, the 48-well plate coated with growth factor-reduced Matrigel (150 μl/well) was allowed to polymerize at 37°C for 60 min. Cells ($3\times10^4$ cells/well) pretreated with arsenic for 24 h were then plated onto the well containing polymerized Matrigel. After 12 h of incubation, the morphology of cells was imaged using a Nikon Eclipse 80i microscope (Tokyo, Japan). The degree of tube formation in each group was estimated with the presence of total length analyzed by ImageJ (http://rsbweb.nih.gov/ij/).

### Knockdown of Id1 protein expression

The short hairpin RNAs (shRNAs) targeting human *Id1* (#1, 5’-CCTACTAGTCACCAGAGACTT-3’; #2, 5’-CTACGACATGAACGGCTGTTA-3’) were cloned into a pLKO.1 vector (*Id1*-pLKO.1) derived from the National RNAi Core Facility (Academia Sinica, Taiwan). A parental pLKO.1 vector without shRNA sequence was used as an empty vector control. Lentiviruses were prepared by transfecting three plasmids (the packing plasmid pCMV8.91, the envelope plasmid pMD.G, and either the shRNA plasmid *Id1*-pLKO.1 or the control plasmid pLKO.1) into 293T cells using Lipofectamine 2000 (Invitrogen, CA, USA) as described previously.[31] MS1 cells were infected with the lentiviral particles containing either *Id1*-pLKO.1 or pLKO.1 collected from the corresponding cell culture medium.

### In vivo angiogenic Matrigel plug assay

For the *in vivo* Matrigel plug experiment in Fig. 1A, female immunodeficient mice (Foxn1$^{nu}$/Foxn1$^{nu}$) were injected subcutaneously with 500 μL Matrigel containing basic FGF (1 ng/mL), heparin (10 U/mL), and either arsenic (0.5 μM) or phosphate buffered saline (PBS) as vehicle control. After 14 days, the mice were sacrificed and Matrigel plugs were dissected out for the quantitation of hemoglobin by Drabkin’s reagent (Sigma-Aldrich) according to the manufacturer’s instructions. As to a separate experiment of the *in vivo* Matrigel plug assay in Fig. 4 C and D, the procedures were identical as described above except for that the Matrigel prepared likewise was additionally mixed with either $1\times10^6$ Id1-knockdowned MS1 cells (Id1-KD), or with the same number of empty vector control MS1 cells (EV). The mice experiments carried out in this study were approved by the Institutional Animal Care and Use Committee of Kaohsiung Medical University.

### In vivo angiogenesis in zebrafish model

The *in vivo* angiogenic assay using embryos of zebrafish was carried out according to a previous report.[32] In brief, approximately 100 embryos were generated per pair of zebrafish via natural pairwise mating. The embryos were then incubated with the indicated concentrations of arsenic at 27°C. After 72 h post-fertilization (hpf), the larvae were anesthetized with 0.5 g/L ethyl 3-aminobenzoate methanesulfonate (Sigma-Aldrich) for 30 min and fixed in 4% paraformaldehyde for 2 h, followed by staining for endogenous alkaline phosphatase activity. The branches of sub-intestinal vessel (SIV) were imaged using a Nikon Eclipse 80i microscope. Experiments involving zebrafish in this study were approved by the Institutional Animal Care and Use Committee of Kaohsiung Medical University.

### Nitric oxide formation assay

The nitric oxide formation was estimated by the method of Griess Reagent according to the procedures of Total Nitric Oxide and Nitrate/Nitrite Parameter Assay Kit (R&D Systems, MN, USA) and expressed as total nitrite/nitrate concentrations by comparing to a standard curve.

### Nitric oxide staining

MS-1 cells in 12-well plates were treated with or without 0.5 μM As for 4 h followed by FA-OMe (10 μM) staining for 8 h according to a previous report[33] and MitoTracker Red (Life Technologies) staining according to the manufacturer's instructions. Images were photographed using Multiphoton and Confocal Microscope System (Leica, Germany) (FA-OMe ex/em: 460/524 nm; MitoTracker Red ex/em: 579/599 nm).

### Statistical analysis

Quantitative data were presented as mean±SD. Two-sided Student's *t* test or one-way ANOVA with post hoc Dunnett's test was used to determine the significant difference between different groups. $P < 0.05$ was considered statistically significantly different from at least three independent experiments.

## Results

### Effects of arsenic on angiogenesis and Id1 expression

The effect of arsenic on cell viability was analyzed by XTT assay in mouse pancreatic endothelial cells (MS1) and human umbilical vein endothelial cells (HUVEC). As shown in Suppl. Fig. 1A, low concentrations of arsenic (0.5–1 μM) promoted cell viability, while that was inhibited by high concentration of arsenic (10 μM). The results suggested that there was a biphasic effect of arsenic on the endothelial cell viability.

To evaluate angiogenic activity of arsenic, the *in vitro* tube formation assay was employed. As shown in Suppl. Fig. 1B and C, low concentrations of arsenic (0.5–1 μM) increased the tube formation, whereas high concentration of arsenic (10 μM) reduced the tube formation in both MS1 and HUVEC cells, implying that arsenic also had a biphasic effect on the angiogenesis. Together, our data showed agreement with previous reports in that low concentration promoted while high concentration suppressed cell viability and angiogenesis by arsenic in endothelial cells.[34-36]

The effect of arsenic on *in vivo* angiogenesis was examined in a mouse model implanted with Matrigel plug and in a zebrafish model. We observed that the level of hemoglobin from the plug containing arsenic in mice was significantly higher than that from the control plug (Fig. 1A), suggesting that new vascular formation was potentiated in the presence of arsenic. The other approach using zebrafish as a model showed the number of branches of sub-intestinal vessel (SIV) increased at 5–10 μM arsenic treatment and decreased at 100–200 μM arsenic treatment (Fig. 1B), in agreement with a recent paper that demonstrated a perturbed vascular development at high dose arsenic treatment.[37]

Since Id1 plays a regulatory role for tumor angiogenesis,[26-28] we next examined the role of Id1 and involving signaling in the arsenic-promoted angiogenesis. As shown in Fig. 2A and B, low concentrations of arsenic (0.5–1 μM) induced Id1 protein expression in MS1 cells. The protein levels of vascular endothelial growth factor (VEGF) were also increased under arsenic treatment (Fig. 2A). In addition, the phosphorylation of Akt and NF-κB (p65 subunit) were both enhanced by arsenic (Fig. 2A), while the expression of total form and phosphorylated STAT3, a transcription activator that may participate in tumor angiogenesis,[38] was unaffected in the presence of arsenic (Suppl. Fig. 2A). The arsenic-induced Id1 expression and Akt phosphorylation were also observed in an *in vivo* zebrafish model (Fig. 1C).

## Involvement of PI3K/Akt and NF-κB in arsenic-induced Id1 expression and tube formation

The involvement of PI3K/Akt and NF-κB in arsenic-induced Id1 expression was further examined by wortmannin, a PI3K/Akt inhibitor, and by QNZ, an inhibitor of NF-κB. As shown in Fig. 2C, the arsenic-induced Akt phosphorylation was inhibited in MS1 cells treated with wortmannin. Notably, the Id1 expression induced by arsenic was suppressed in the presence of wortmannin (Fig. 2C) or QNZ (Fig. 2D). Moreover, the arsenic-promoted *in vitro* tube formation was inhibited in the presence of wortmannin (Fig. 2E) or QNZ (Fig. 2F). These data suggested that activation of PI3K/Akt and NF-κB signaling might play a regulatory role in the arsenic-induced Id1 expression and angiogenesis.

### Involvement of nitric oxide synthase (NOS) in arsenic-induced Id1 expression and tube formation

Although the effect of arsenic on nitric oxide production[35, 39] and eNOS activation[40] were reported, the role of nitric oxide in Id1-mediated angiogenesis induced by arsenic has not been elucidated before. In the present study, MS1 cells treated with arsenic consistently showed an increased nitric oxide formation (Fig. 3A and Suppl. Fig. 2B), and treatment with DETA-NONOate, a nitric oxide donor, was able to upregulate the Id1 expression and *in vitro* tube formation in these cells (Fig. 3B, C), implying that nitric oxide might play a role upstream of Id1 in this process. Furthermore, we found that treatment of arsenic increased the protein expression of phospho-eNOS (Ser1177) and iNOS (Fig. 3D). The involvement of nitric oxide in arsenic-stimulated Id1 expression and tube formation were further examined by two nitric oxide synthase inhibitors, L-NAME and 1400W. As shown in Fig. 3E and 3F, the arsenic-induced Id1 expression was suppressed in the presence of L-NAME and 1400W, respectively. In addition, the arsenic-promoted tube formation was inhibited by L-NAME (Fig. 3E) or 1400W (Fig. 3F). Collectively, the data suggested that the arsenic-induced Id1 expression and angiogenesis were mediated through a signaling pathway involving nitric oxide.

### Id1 as the downstream effector for arsenic-induced angiogenesis

To further investigate the role of Id1 in arsenic-induced angiogenesis, the endogenous expression of Id1 was knockdowned by a lentiviral shRNA approach (Fig. 4A). As shown in Fig. 4A and B, the arsenic-induced *in vitro* tube formation was suppressed in the Id1-knockdowned (Id1-KD) MS1 cells. In addition, the level of arsenic-promoted *in vivo* angiogenesis was reduced in the presence of Id1-KD cells (Fig. 4C and D). Together, the results suggested that Id1 acted as an important factor mediating the arsenic-promoted angiogenesis.

## Discussion

Previous studies showed that exposure to arsenic was correlated with increasing risks of various carcinogenesis.[8, 24, 41-45] In addition to the tumorigenic effect, the angiogenic activity of arsenic was also reported. For example, it was reported that low level of arsenic promoted neovascularization and blood vessel remodeling.[24, 25] Treatment with arsenic also stimulated cell migration and tube formation of human microvascular endothelial cells.[46] Moreover, the level of tumor angiogenesis was elevated by arsenic in mice xenografted with human adenocarcinoma cells.[23] In the present study, we consistently showed that low arsenic promoted endothelial cell viability and angiogenesis (Suppl. Fig. 1), whereas the opposite effect of arsenic at a higher concentration which caused the reduction of angiogenesis may be explained by its cytotoxic effect at such amount of arsenic.[35, 36, 47, 48]

It was reported that PI3K/Akt signaling played an important role in the arsenic-induced angiogenesis.[49-51] Exposure to arsenic stimulated the PI3K/Akt phosphorylation cascade and resulted in cellular transformation characterized by increases of proliferation and anchorage-independent growth.[52] Arsenic activated MAPK and PI3K/Akt pathways that were required for the arsenic-induced expression of COX-2, HIF-1α and VEGF.[53, 54] Our results suggested a novel regulatory role of PI3K/Akt in the arsenic-induced Id1 expression and tube formation (Fig. 2C and E).

The possible involvement of NF-κB, a downstream effector of PI3K/Akt in endothelial cells,[55-57] was further investigated in this study. We found that arsenic was able to trigger NF-κB activation (Fig. 2A) as reported previously.[47, 58] Notably, the arsenic-enhanced Id1 expression and tube formation were suppressed in the presence of QNZ (Fig. 2D and F). We noticed that angiogenesis-related genes driven by NF-κB, such as IL-8 and Col1, were reportedly unnecessary for the arsenic-induced tube formation.[59] Therefore, it would be worthwhile to determine whether there are specific NF-κB-regulated genes in the Id1-mediated angiogenesis induced by arsenic.

Previous report showed that the level of *in vivo* angiogenesis was reduced to a lesser degree in Id1$^{-/-}$ mice in contrast to that in Id1$^{+/+}$ mice.[28] In addition, Id1$^{-/-}$ mice xenografted with human lung carcinoma cells had defects of microvascular formation on the implanted site, suggesting that Id1 might be a major effector for the tumor angiogenic activity.[28] Strong Id1 expression was also observed in the surrounding blood vessels of high-grade glioma tumor tissues, while only weak Id1 expression was observed in those of low-grade tumor tissues.[27] Our current data unveiled an angiogenic role of Id1 in the vascular endothelial cells when treated with arsenic. For instance, the arsenic-induced *in vitro* tube formation was significantly reduced in the Id1-knockdowned MS1 cells compared with the control cells (Fig. 4A and B). Furthermore, the arsenic-induced *in vivo* angiogenic activity was suppressed in the mice xenografted with Id1-KD cells *versus* the mice xenografted with control cells (Fig. 4C and D). As angiogenesis is an essential event for tumor growth and metastasis, the Id1-mediated tumor angiogenesis may be developed into a therapeutic potential against cancer progression.[15, 20, 60]

While nitric oxide is a known factor involving in arsenic-induced angiogenesis,[61, 62] the role of nitric oxide in arsenic-induced Id1 expression has not been elucidated. Several lines of evidence in our current study suggested the possible involvement of nitric oxide signaling and Id1 in these arsenic-induced events, including 1) the level of nitric oxide formation was increased by arsenic (Fig. 3A), 2) Id1 expression and tube formation were enhanced in the presence of DETA-NONOate, a known NO donor

(Fig. 3B and C), and 3) application of the NOS inhibitor L-NAME and 1400W showed inhibitory effect on Id1 expression and tube formation induced by arsenic (Fig. 3E and F). It was reported that NOS activities were regulated downstream of PI3K/Akt and NF-κB signaling.[63-66] Intriguingly, a mutual regulation might be present between Id1 and PI3K/Akt.[51, 67-69] Therefore, it will be valuable to investigate the complex connection of these signaling molecules involved in the arsenic-induced angiogenesis (Fig. 4E).

In summary, our data showed for the first time that Id1 mediated arsenic-promoted angiogenesis. In addition, PI3K/Akt, NF-κB and nitric oxide had regulatory roles in this process. The current results may hence further our understanding towards the role of Id1 in arsenic-associated angiogenesis and suggest for its potential application as a target of antiangiogenesis therapy in arsenic-associated cancer.

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## Figure legends

**Fig. 1** Effects of arsenic on *in vivo* angiogenesis. (A) Matrigels containing bovine FGF (1 ng/mL), heparin (10 U/mL), and either with or without arsenic (0.5 μM) were injected subcutaneously to the female immunodeficient mice. After 14 days, the Matrigel plugs were dissected out and the hemoglobin levels were determined by Drabkin's reagent. (B) Embryos of zebrafish were incubated with different doses of arsenic for 72 h. After anesthetization, the larvae were stained and imaged, and the number of branches of sub-intestinal vessel (SIV) was quantitated. Arrows showed typical appearance of the SIV branches under different conditions of arsenic treatment. *Significant difference of $P < 0.05$ compared with the untreated control by Student's *t* test. (C) Arsenic induced protein expression of Id1 and phospho-Akt (Ser473) in zebrafish. Embryos of zebrafish were incubated either with or without arsenic (10 μM) for 72 h. After anesthetization, the total proteins of larvae were extracted and subjected to Western blot analysis.

**Fig. 2** Involvement of PI3K/Akt signaling in arsenic-induced Id1 protein expression and *in vitro* angiogenesis. (A) MS1 cells were treated with arsenic (0.5 μM) for the indicated lengths of time, followed by Western blot analysis for protein expression of VEGF, phospho-Akt (Ser473), Akt, phospho-p65 (Ser536), p65, and Id1. (B) MS1 cells were treated with different doses of arsenic for 24 h, followed by Western blot analysis for Id1 protein expression. (C) MS1 cells were pretreated with or without the PI3K/Akt inhibitor wortmannin (1 μM) for 2 h, followed by arsenic treatment (0.5 μM) for 24 h. The protein expression of phospho-Akt (Ser473) and Id1 was analyzed by Western blot. (D) MS1 cells were pretreated with or without NF-κB inhibitor QNZ (10 nM) for 2 h, followed by arsenic treatment (0.5 μM) for 24 h. The protein expression of Id1 was analyzed by Western blot. (E) MS1 cells were pretreated with or without wortmannin (1 μM) for 2 h, followed by *in vitro* tube formation assay in the presence or absence of arsenic (0.5 μM) for 24 h. (F) MS1 cells were pretreated with or without QNZ (10 nM) for 2 h, followed by *in vitro* tube formation assay in the presence or absence of arsenic (0.5 μM) for 24 h. *Significant difference of $P < 0.05$ by one-way ANOVA with post hoc Dunnett's test.

**Fig. 3** Involvement of nitric oxide in arsenic-induced Id1 protein expression and *in vitro* angiogenesis. (A) MS1 cells were treated with different doses of arsenic for 48 h, and the cell culture media were collected for the nitric oxide formation assay. (B) MS1 cells were treated with different doses of DETA-NONOate for 24 h, followed by Western blot analysis for Id1 protein expression. (C) MS1 cells were treated with DETA-NONOate (10 μM) for 24 h, followed by the *in vitro* tube formation assay. *Significant difference of $P < 0.05$ by Student's $t$ test. (D) MS1 cells were treated with arsenic (0.5 μM) for 24 h, followed by Western blot analysis for protein expression of phospho-eNOS (Ser1177), eNOS, iNOS and Id1. (E) MS1 cells were pretreated with or without the nitric oxide synthase inhibitor L-NAME (100 μM) for 2 h, followed by the presence or absence of arsenic treatment (0.5 μM) for 24 h. The protein expression of Id1 and tube formation ability were analyzed. (F) MS1 cells were pretreated with or without the nitric oxide synthase inhibitor 1400W (10 μM) for 2 h, followed by the presence or absence of arsenic treatment (0.5 μM) for 24 h. The protein expression of Id1 and tube formation ability were analyzed *Significant difference of $P < 0.05$ by one-way ANOVA with post hoc Dunnett's test.

**Fig. 4** Involvement of Id1 in the arsenic-promoted angiogenesis. (A) Protein expression levels of Id1 in the Id1-knockdowned (Id1-KD#1 and Id1-KD#2) and empty vector control (EV) MS1 cells were assessed by Western blot. As a result, Id1-KD#2 MS1 cells were chosen for the experiments in (B)–(D). (A, B) Id1-KD and EV MS1 cells were treated with or without arsenic (0.5 μM) for 24 h, followed by the in vitro tube formation assay. (C, D) Id1-KD or EV MS1 cells were mixed with the Matrigel containing bovine FGF (1 ng/mL), heparin (10 U/mL), and either with or without arsenic (0.5 μM), followed by subcutaneous injection to immunodeficient mice for the Matrigel plug assay. (E) Schematic representation of a summary for the current work. The flow chart shows that arsenic-induced angiogenesis is mediated through Id1 expression regulated via PI3K/Akt, NF-κB and nitric oxide signaling. The dashed line suggests that a mutual regulation between Id1 and PI3K/Akt may possibly exist in this process (see Discussion). Wortmannin, PI3K/Akt inhibitor; QNZ, NF-κB inhibitor; L-NAME and 1400W, nitric oxide synthase inhibitors. *Significant difference of $P < 0.05$ by one-way ANOVA with post hoc Dunnett's test.

**Suppl. Fig. 1** Effects of arsenic on endothelial cell viability and *in vitro* angiogenesis. (A) MS1 and HUVEC cells were treated with different doses of arsenic for 72 h. The number of viable cells was determined by XTT assay. (B) MS1 and HUVEC cells were treated with different doses of arsenic for 24 h, followed by *in vitro* tube formation assay. (C) Quantification of the total tube lengths from the corresponding groups in (B). *Significant difference of $P < 0.05$ compared with the untreated control by Student’s $t$ test.

**Suppl. Fig. 2** Effects of arsenic on the protein expression STAT3 and nitric oxide in MS1 endothelial cells. (A) Cells were treated with arsenic (0.5 μM) for the indicated lengths of time, followed by Western blot analysis. (B) MS1 cells were treated with or without arsenic (0.5 μM), followed by nitric oxide staining (FA-OMe, green in color) and mitochondrial staining (Mitochondrial, red in color). Dashed boxes were enlarged in the bottom of each fluorescent micrograph.

Fig. 1

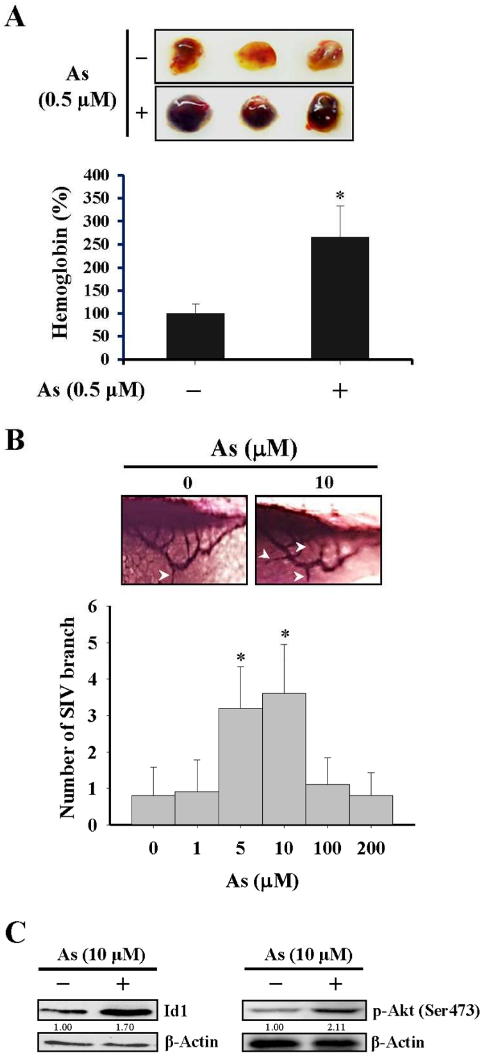

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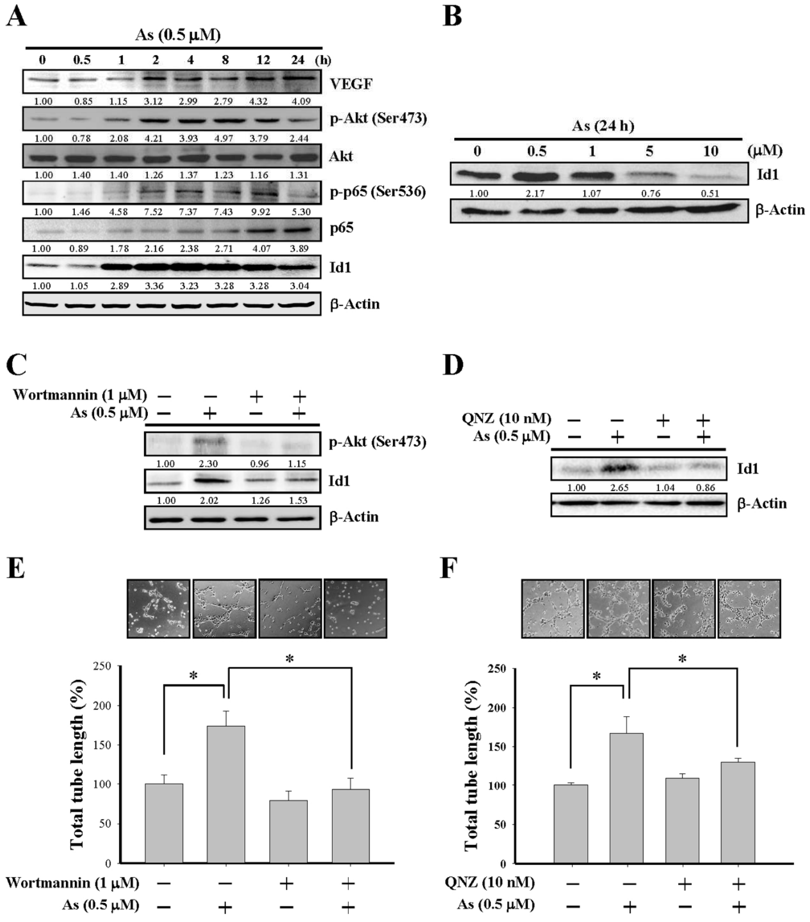
Fig. 2



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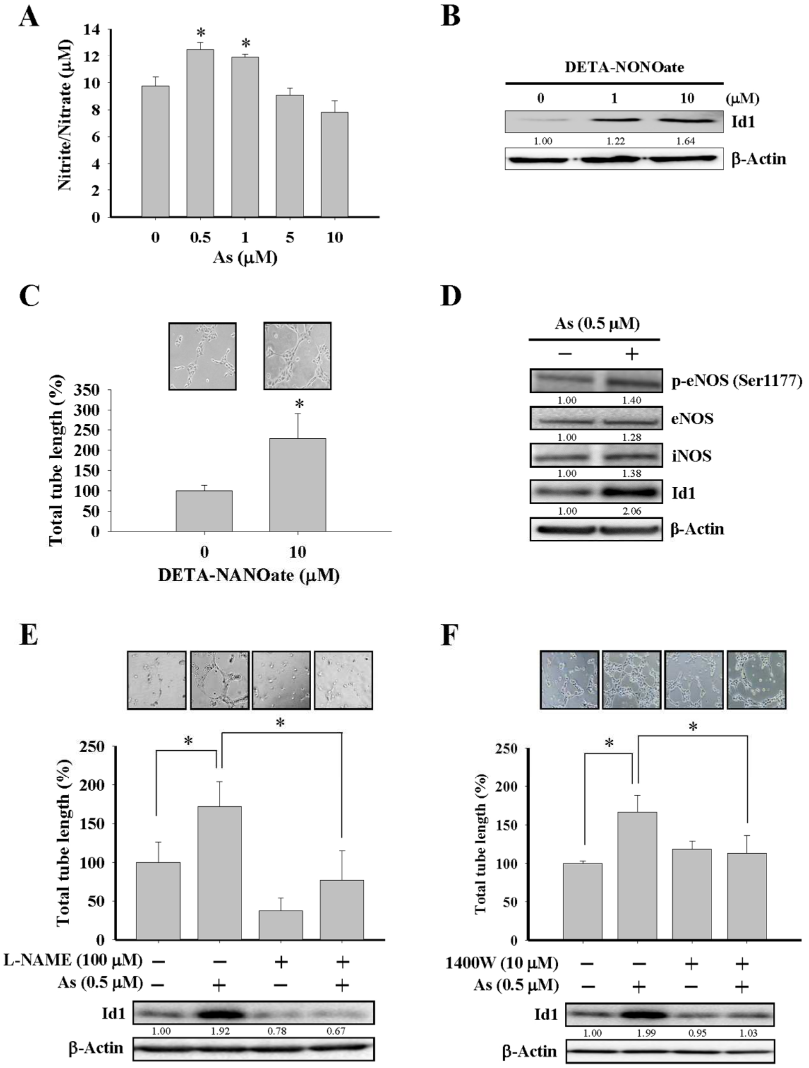

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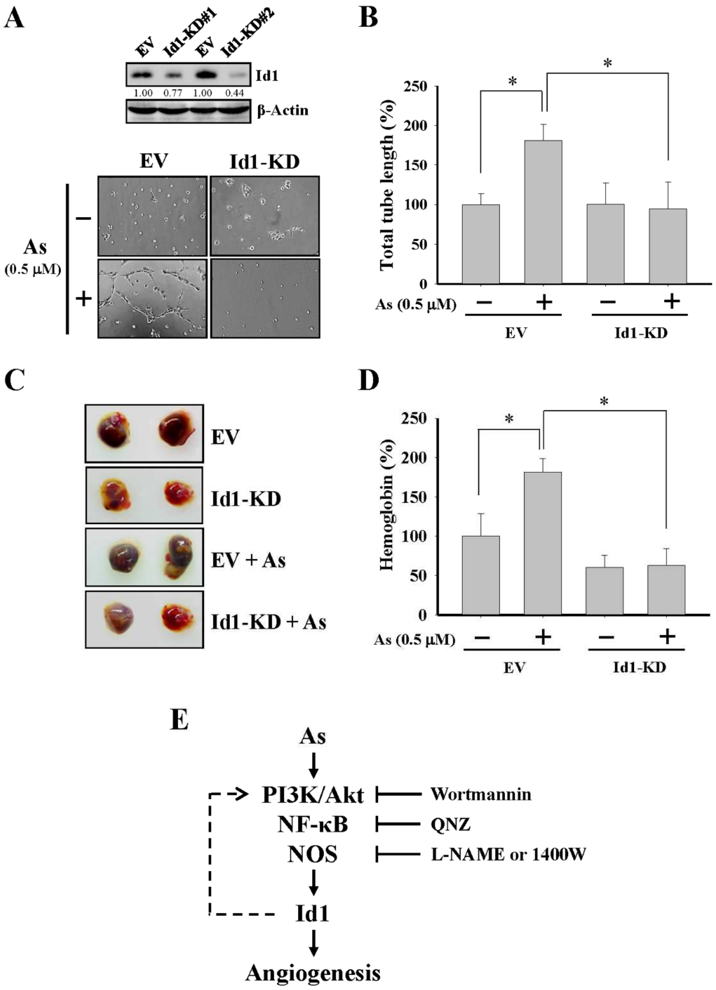

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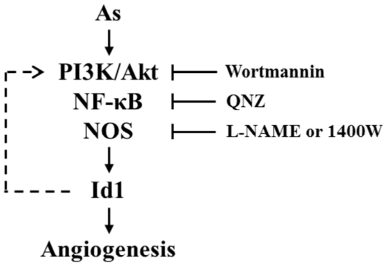

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