4.1N suppresses hypoxia-induced epithelial-mesenchymal transition in epithelial ovarian cancer cells

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Received January 18, 2015; Accepted October 29, 2015

DOI: 10.3892/mmr.2015.4634

Abstract. Protein 4.1N (4.1N) is a member of the protein 4.1 family and is essential for the regulation of cell adhesion, motility and signaling. Previous studies have suggested that 4.1N may serve a tumor suppressor role. However, the molecular mechanisms remain unclear. In the current study, the role of 4.1N in the downregulation of hypoxia-induced factor 1α (HIF-1α) under hypoxic conditions and therefore the suppression of hypoxia induced epithelial-mesenchymal transition (EMT) was investigated. The data were obtained from overexpressed and knockdown 4.1N epithelial ovarian cancer (EOC) cell lines. It was identified that 4.1N was capable of regulating the sub-cellular localization and expression levels of HIF-1α, by which 4.1N served a dominant role in the suppression of hypoxia-induced EMT and associated genes. Collectively, the data of the current study identified 4.1N as an inhibitor of hypoxia-induced tumor progression in EOC cells and highlighted its potential role in EOC therapy.

Introduction

Epithelial ovarian cancer (EOC) is a gynecological cancer associated with high mortality worldwide (1). In the USA, the number of newly diagnosed cases and the cases of EOC-associated mortality were 21,980 and 14,270, respectively, in 2014 (2). Although it has been previously demonstrated that a variety of non-specific symptoms prior to diagnosis occur in the majority of patients, early diagnosis remains a challenge (3). Furthermore, peritoneal dissemination, which is induced by hypoxic stress resistance, contributes substantially to the mortality rate of patients with EOC (4-7). Thus, it is essential to understand the molecular mechanisms of hypoxia-induced EOC progression.

4.1N, the protein product of the EPB41L1 gene, is a member of the protein 4.1 family (8). The members of the protein 4.1 family include 4.1R (9), 4.1B (10), 4.1G (11) and 4.1N (12). Protein 4.1 family members connect the actin cytoskeleton to various transmembrane proteins (12) and serve important roles in cell morphogenesis, membrane structure and cell adhesion (13). Recently, the roles of protein 4.1 family members in growth regulation and tumor development have become increasingly recognized, and a loss of 4.1B and 4.1R expression has been reported in lung, breast, prostate, ovary and brain cancer, and meningioma (14-16). However, the role of 4.1N in cancer remains to be fully elucidated. It was previously reported that defective expression of 4.1N was correlated with tumor progression, aggressive behavior and chemotherapy resistance in EOC (17). Experiments on nude mice (unpublished data) have demonstrated that 4.1N may significantly inhibit the ability of peritoneal dissemination of EOC cells.

Hypoxia contributes to enhanced invasiveness, angiogenesis and distant metastasis in various tumor types (18). Previous studies have indicated that hypoxia serves an important role in the initiation of peritoneal dissemination of EOC cells (8,18,19). The most important regulator under low levels of oxygen is hypoxia-inducible factor-1 (HIF-1), which is comprised of the HIF-1α and HIF-1β subunits (20). Hypoxic conditions activate HIF-1α during numerous critical behaviors of cancer progression, including angiogenesis (21,22), energy metabolism (23), resistance to radiation therapy and chemotherapy (24,25) and epithelial-mesenchymal transition (EMT) (26-28). In cancer, EMT serves an important role in tumor progression and is marked by a loss of epithelial features, particularly loss of E-cadherin and an upregulation of mesenchymal properties (29-31). HIF-1α is an important factor in controlling the expression of certain EMT regulators, including Snail, Twist and lysyl oxidase (LOX), all of which are involved in various EMT processes occurring during embryogenesis and tumorigenesis (7,27,32,33).

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Key words: epithelial ovarian cancer, 4.1N, EMT, hypoxia, HIF-1α

The current study hypothesized that 4.1N may suppress hypoxia-induced EMT in EOC cells via regulating the expression levels and subcellular localization of HIF-1α.

Materials and methods

Cell culture. A2780 and SKOV-3 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.), respectively with 10% fetal bovine serum (FBS; Yuanheng JInma Biotechnology Co. Ltd., Beijing, China). Cells were cultured with 2 mg/ml $NaHCO_3$ at 37˚C in a humidified chamber with 5% CO_2. To mimic hypoxic conditions, cells were starved with 0.5% FBS and then were treated with $CoCl_2$ (Aladdin Industrial, Inc., Shanghai, China) at 250 μmol for different lengths of time. Cells were treated for a different length of time to induce HIF-1α expression due to the diverse characteristics of A2780 (12 and 24 h) and SKOV-3 (48 and 72 h) cells.

Cell transfection and RNA interference. A2780 cells were transfected with pEGFP-4.1N [provided by Dr Xiuli An (Red Cell Physiology Laboratory, New York Blood Center, New York, NY, USA) with sequencing identification being performed in Gynecological Oncology Laboratory, Department of Pathology, Peking University, Beijing, China] or pEGFP-3C with Lipofectamine® 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Human 4.1 short hairpin RNA (shRNA) was obtained from Shanghai GenePharma Co. Ltd. (Shanghai, China). The target sites for 4.1N shRNA were 5'-GCAACATCACTCGAAATAA-3' (sh4.1N-1) and 5'-ACGGAAATCCGTTCTCTTT-3' (sh4.1N-2). For knocking down 4.1N, two independent experiments with the above two shRNAs were conducted and the efficiency of interference was confirmed. A scrambled shRNA 5'-TGTTCGCATTATCCGAACCAT-3' was used as a negative control. The cell line (SKOV-3) that endogenously expressed 4.1N was transfected with different shRNA constructs to evaluate the effects on tumor cells. Subsequent to incubation at 37˚C for 24 h, G418 (800 μg/ml; Invitrogen; Thermo Fisher Scientific, Inc.) was applied to stably screen and isolate the resistant colonies.

Cell viability assay. The cells were seeded (3,000 cells/well) into 96-well plates and were incubated at 37˚C with 5% carbon dioxide in the presence of $CoCl_2$ (200, 250, 300 and 0 μmol cell control) for different lengths of time. The negative control group underwent the same procedures, however the cells were not plated. At each end point, a batch of cells was stained with 20 μl sterile 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) at 37˚C for 4 h. The culture medium was then removed and 150 μl dimethyl sulfoxide (Sigma-Aldrich) was added and thoroughly mixed for 10 min. The cell viability assay was applied to spectrometric absorbance at 490 nm and was measured using a microplate reader (Thermo Fisher Scientific, Inc.). The cell viability rate was calculated as (treatment group-negative control group)/(cell control group-negative control group). Each group contained six wells and the experiments were performed in triplicate.

Western blotting analysis. Cells were lysed in radioimmunoprecipitation assay lysis buffer supplemented with a protease inhibitor cocktail (Applygen Technologies, Inc., Beijing, China). The cells were then centrifuged at 10,000 x g for 10 min at 4˚C. The supernatant was collected, protein (30 μg) was denatured in sodium dodecyl sulfate (SDS) sample buffer (Applygen Technologies, Inc.) at 100˚C for 5 min and separated by electrophoresis on a 10% SDS-polyacrylamide gel electrophoresis (Applygen Technologies, Inc.), then transferred to a nitrocellulose membrane (Applygen Technologies, Inc.). The membranes were blocked in Tris-buffered saline with Tween-20 (TBST) with 5% non-fat milk for 1 h at 37˚C, then were incubated with the following primary antibodies: rabbit monoclonal E-cadherin (cat. no. 1702-1; 1:2,000) and rabbit polyclonal N-cadherin (cat. no. 21474; 1:500, purchased from Epitomics, Inc. (Burlingame, CA, USA), rabbit polyclonal HIF-1α (cat. no. 3716; 1:1,000) from Cell Signaling Technology, Inc. (Danvers, MA, USA) and rabbit anti-4.1N (donated by Dr Xiuli An; 1:500) in blocking buffer (Applygen Technologies, Inc.) overnight at 4˚C. The membranes were washed three times in TBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody (cat. no. ZB-2301; 1:2,000) or anti-mouse (cat. no. ZB-2305; 1:2,000) antibodies (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) for 1 h at room temperature and then visualized by enhanced chemiluminescence (ECL) using a SuperEnhanced Chemiluminescence detection kit (Applygen Technologies, Inc.). The Gel Doc™ XR+ imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used for capturing the images.

Immunofluorescence. Cells grown on glass cover-slips were fixed with 4% paraformaldehyde for 10 min at room temperature. Subsequent to washing in phosphate-buffered saline (PBS; GE Healthcare Life Sciences, Chalfont, UK), the cells were incubated with the blocking reagent [1% horse serum albumin (Gibco, Thermo Fisher Scientific, Inc.) in PBS] for 15 min. Cells were incubated with rabbit anti-HIF-1α (cat. no. ZA-0552; 1:1,000; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) at 4˚C overnight. The primary antibody was omitted for the negative control slides. Subsequent to washing, samples were incubated with Alexa Fluor 594-conjugated affinipure goat anti-rabbit IgG (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) for 1 h at 37˚C. Subsequent to staining with 6-diamino-2-phenylindole (Cell Signaling Technology, Inc.), the cells were examined under a microscope (U-TV0.5XC-3; Olympus, Tokyo, Japan).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA was synthesized using the FastQuant RT kit [Tiangen Biotech (Beijing) Co., Ltd., Beijing, China]. cDNA (10 ng) was then used for the RT-qPCR reaction using SuperReal PreMix Plus [Tiangen Biotech (Beijing) Co., Ltd.]. RT-qPCR was performed using an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.). The results were

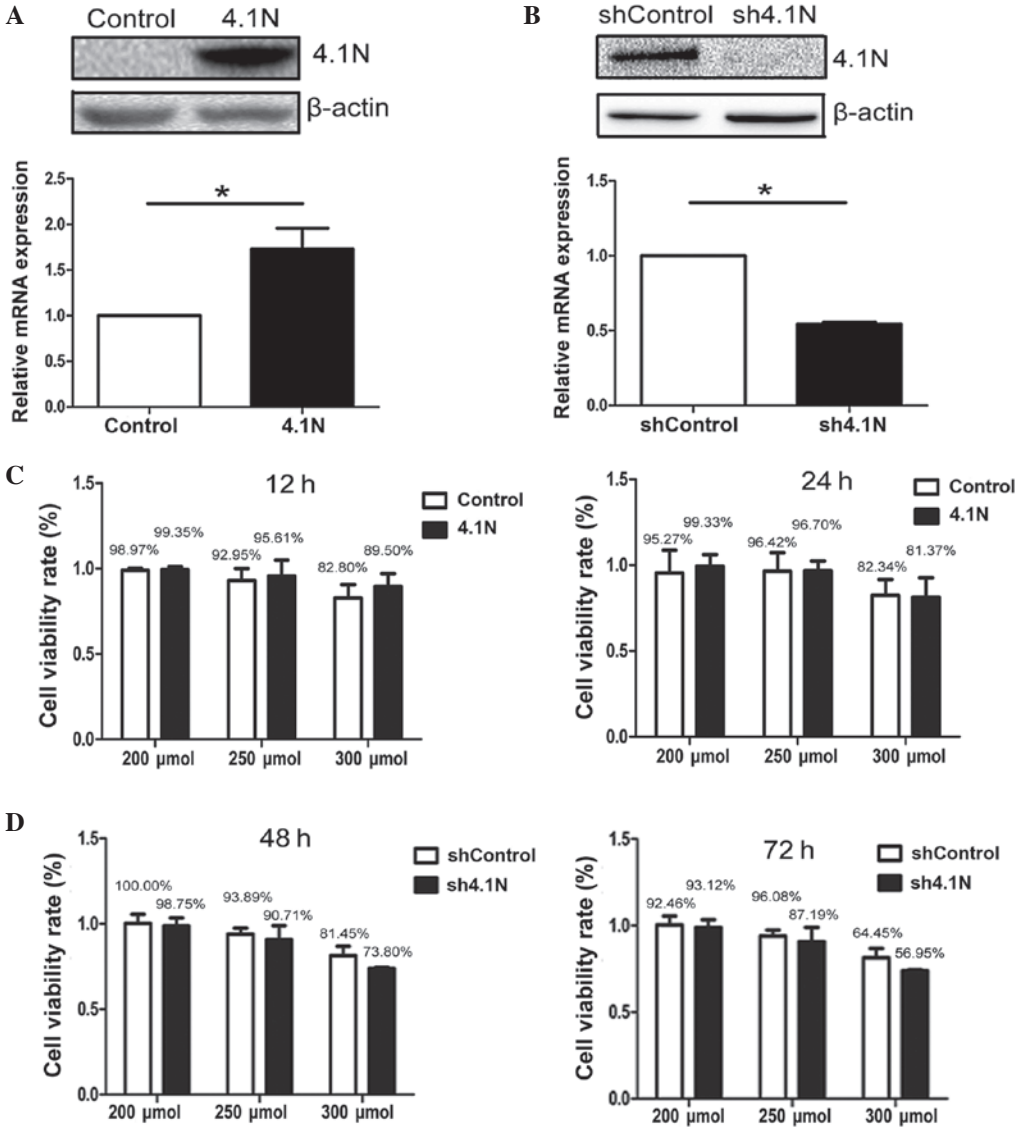

Figure 1. Overexpression of 4.1N in A2780 cells, knockdown of 4.1N in SKOV-3 cells and cell viability assay. (A) A2780 cells were transfected with the empty vector or 4.1N, then were subjected to western blotting and RT-qPCR analysis. (B) Knockdown of 4.1N using shRNA (sh4.1N) was performed in SKOV-3 cells. Scrambled shRNA (shControl) was used as the negative control. Western blotting and RT-qPCR analysis for the expression levels of 4.1N was conducted. (C and D) Cell viability assay of A2780 and SKOV-3 cells with 12 and 24 h treatment with $CoCl_2$ at 200, 250 and 300 μmol and with controls for A2780 cells and 48 and 72 h for SKOV-3 cells. Values are presented as the mean ± standard deviation of triplicate measurements. *P<0.05. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; shRNA, short hairpin RNA.

calculated by the $2^{-(\Delta\Delta Ct)}$ method. The primer sequences were as follows: 4.1N, forward 5'-AGGAAACCACGCCGAGACACAA-3' and reverse 5'-GGTGGATGAGTTTGCTGTTGGG-3'; Twist, forward 5'-GACAGTGATTCCCAGACGG-3' and reverse 5'-GTCCATAGTGATGCCTTTCCT-3'; Snail, forward 5'-TCGGAAGCCTAACTACAGCG-3' and reverse 5'-GATGAGCATTGGCAGCGAG-3'; HIF-1α, forward 5'-CTGAGGTTGGTTACTGTTGGTATC-3' and reverse 5'-AGTGTACCCTAACTAGCCGAGGAA-3'; LOX, forward 5'-ATGGTGCTGCTCAGATTTCC-3' and reverse 5'-TGACAACTGTGCCATTCCCA-3'. The cycling conditions were 95˚C for 15 min, followed by 40 cycles at 95˚C for 10 sec, 55˚C for 20 sec and 72˚C for 30 sec.

Statistical analysis. All values are presented as the mean ± standard deviation and are representative of an average of a minimum of three independent experiments. Student's t-test or analysis of variance for unpaired data was used to compare the mean values using GraphPad Prism software, version 5 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

4.1N inhibits expression and nuclear accumulation of HIF-1α under hypoxia. A previous study indicated that 4.1N protein was absent in A2780 and present in SKOV-3 cells (17). Overexpression of 4.1N in A2780 and knockdown of 4.1N in SKOV-3 cells was confirmed by western blotting and RT-qPCR (Fig. 1A and B), respectively. Subsequently, the role of 4.1N in the regulation of HIF-1α expression was investigated under hypoxia. A cell viability assay was conducted in order to confirm the amount of $CoCl_2$ that could be tolerated by cells.

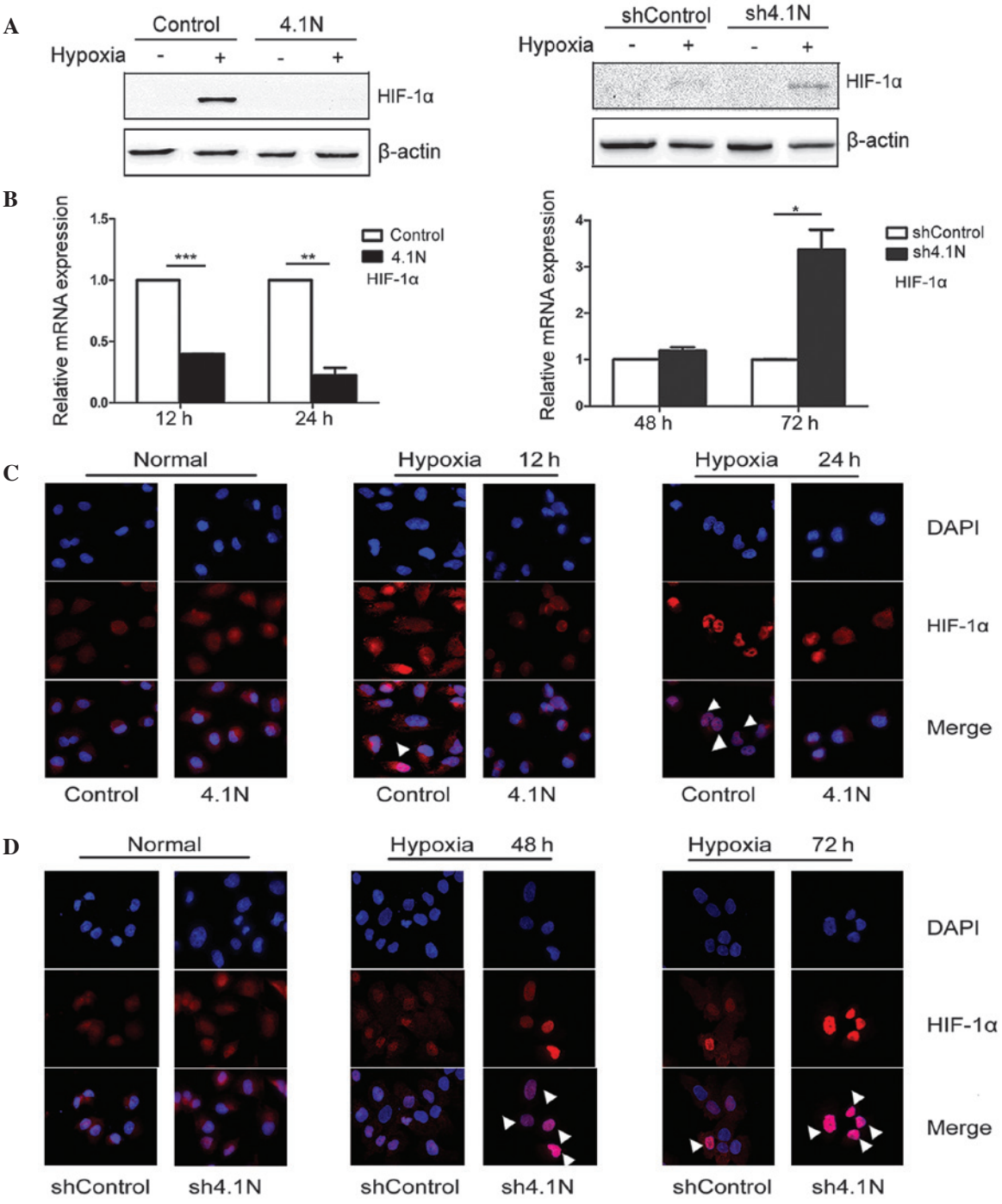

Figure 2. 4.1N inhibits the expression and nuclear accumulation of HIF-1α under hypoxic conditions. (A) Western blot analysis of HIF-1α in A2780 (control and 4.1N) cells and SKOV-3 (shControl and sh4.1N) cells following 24 and 72 h of hypoxia, respectively. (B) Under hypoxic conditions, A2780 (control and 4.1 N) cells were cultured for 12 and 24 h, while SKOV-3 (shControl and sh4.1N) cells were cultured for 48 and 72 h. HIF-1α mRNA levels were analyzed by reverse transcription-quantitative polymerase chain reaction. (C) A2780 cells (4.1N and control) and (D) SKOV-3 cells (shControl and sh4.1N) were cultured under normoxic and hypoxic conditions. Immunofluorescence analysis was performed at (C) 12 and 24 h subsequent to hypoxic induction for A2780 cells and (D) following 48 and 72 h hypoxia for SKOV-3 cells. Arrows indicate co-localization of HIF-1α and 6-diamino-2-phenylindole. Values are presented as the mean ± standard deviation of triplicate measurements. ***P<0.001, **P<0.005, *P<0.05. HIF-1α, hypoxia-inducible factor 1α; sh, short hairpin.

In A2780 cells, the cell viability rate was greater than 90% when treated with $CoCl_2$ at 200 and 250 μmol, however was reduced to less than 90% at 300 μmol. In SKOV-3 cells, due to the longer treatment duration (48 and 72 h), the cell viability rate with $CoCl_2$ at 200 and 250 μmol was lower than that of A2780 cells, however remained greater than 85%. However, with treatment with 300 μmol for 48 and 72 h, viability was reduced to approximately 60% (Fig. 1C and D). In western blot analysis, proteins were collected following 24 and 72 h hypoxia treatment for A2780 cells and SKOV-3 cells, respectively. Under normoxic conditions, the expression levels of HIF-1α were significantly reduced in the presence of 4.1N. By contrast, the amount of HIF-1α protein was markedly increased with the absence of 4.1N (Fig. 2A). RT-qPCR results are presented in Fig. 2B. HIF-1α mRNA expression was significantly downregulated in the presence of 4.1N;

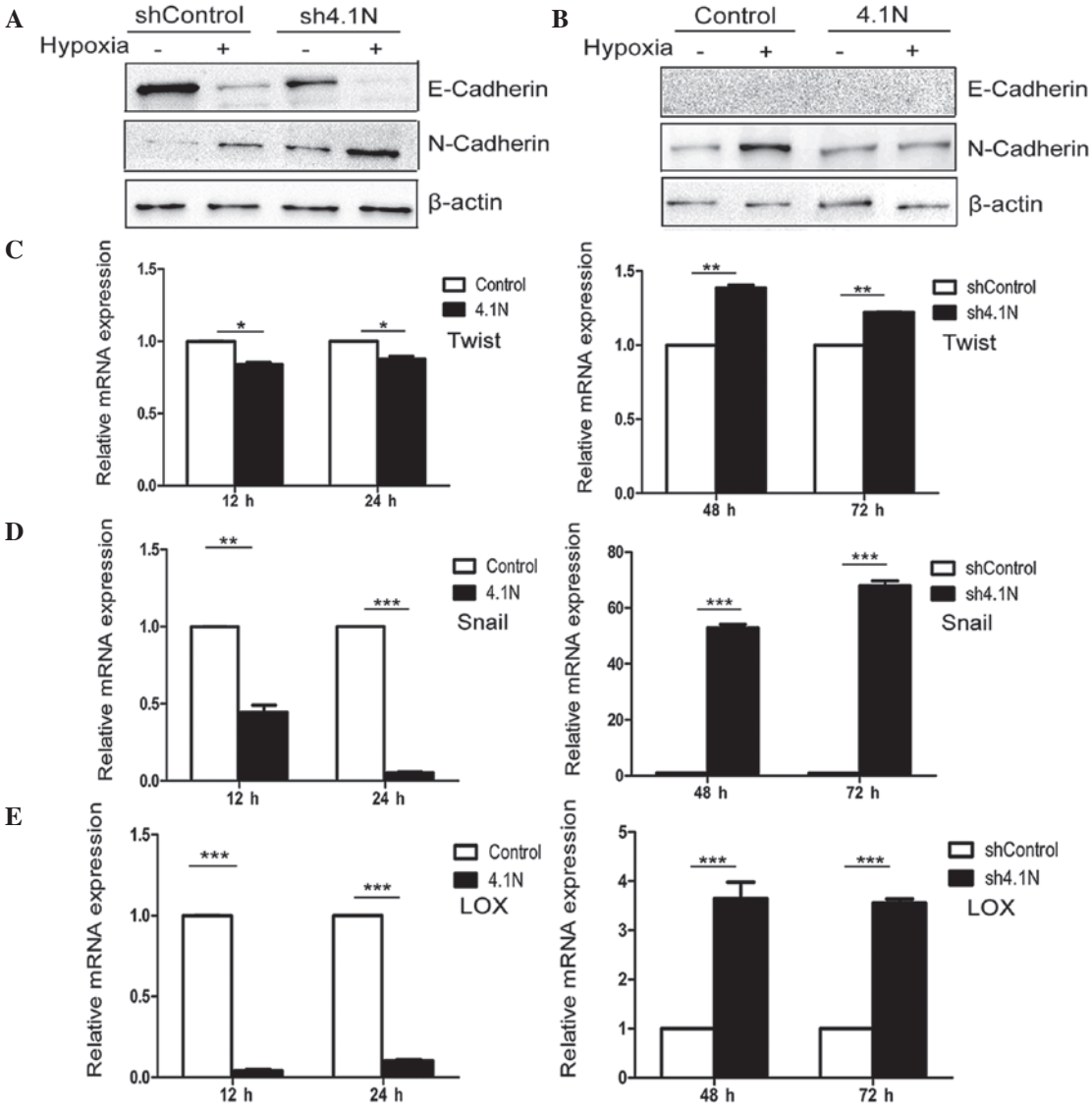

Figure 3. 4.1N affects the expression of E-cadherin and N-cadherin under hypoxia and inhibits the expression of genes that regulate epithelial-mesenchymal transition. Expression of E-cadherin and N-cadherin in (A) SKOV-3 (shControl and sh4.1N) cells subsequent to 72 h of hypoxia and (B) A2780 (control and 4.1N) cells following 24 h of hypoxia were analyzed by western blotting. (C-E) Under hypoxic conditions, A2780 (control and 4.1N) cells were cultured for 12 and 24 h while SKOV-3 (shControl and sh4.1N) cells were cultured for 48 and 72 h. Relative mRNA levels of (C) Twist, (D) Snail and (E) LOX were analyzed by reverse transcription-quantitative polymerase chain reaction. Values are presented as the mean ± standard deviation of triplicate measurements. ***P<0.001, **P<0.005, *P<0.05. sh, short hairpin.

whereas, the stress-response of 4.1N-knockdown cells to hypoxia was significantly increased at 72 h compared with the control. Immunofluorescence staining was then conducted in order to confirm the alterations in HIF-1α subcellular localization under hypoxia. Nuclear accumulation of HIF-1α in 4.1N-overexpressing A2780 cells was observed to be markedly reduced compared with that of control cells under hypoxia, particularly following 24 h of hypoxia (Fig. 2C). In SKOV-3 cells, the absence of 4.1N led to clear nuclear localization of HIF-1α at 48 and 72 h compared with the controls (Fig. 2D). These results suggest that 4.1N may suppress the expression and nuclear localization of HIF-1α under hypoxic conditions.

4.1N inhibits hypoxia-induced EMT. Loss expression of epithelial markers such as E-cadherin and increases in expression of mesenchymal markers such as N-cadherin are typical events of epithelial cells undergoing EMT. It was identified

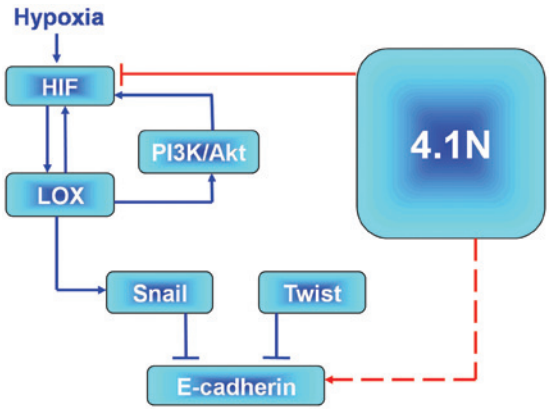

Figure 4. Proposed model for how 4.1N regulates hypoxia-induced epithelial-mesenchymal transition. 4.1N may inhibit HIF and upregulate E-cadherin via the HIF-LOX-Snail-E-cadherin cascade. 4.1N may also suppress HIF or directly interact with E-cadherin. HIF, hypoxia-inducible factor; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; LOX, lysyl oxidase.

that with the absence of 4.1N, the protein levels of E-cadherin were markedly reduced and the protein levels of N-cadherin were upregulated under normoxic and hypoxic conditions, particularly with those with hypoxia (Fig. 3A). When 4.1N was overexpressed, N-cadherin expression was markedly reduced under conditions of hypoxia (Fig. 3B). However, due to the lack of endogenous expression of E-cadherin in A2780 cells, the effect of hypoxia on E-cadherin of A2780 cells in the 4.1N and control groups was not detected.

4.1N suppresses the expression of positive EMT regulators. Hypoxia, an important tumor micro-environmental factor, induces the expression of numerous EMT regulators, including Snail, Twist and LOX (7,33,34). Thus, RT-qPCR was used to observe the transcriptional mRNA levels of Snail, Twist and LOX. Fig. 3C-E indicate that overexpression of 4.1N may significantly inhibit the mRNA expression levels of the three measured EMT regulators under hypoxic stress, while their mRNA expression levels were significantly increased in the EOC cells in the absence of 4.1N under hypoxia.

Discussion

The protein 4.1 family has been observed to serve an important role in the regulation of growth and tumor progression (35). A previous study indicated that 4.1N was a potential tumor suppressor in EOC (17). Previous studies have indicated that peritoneal dissemination, in which EMT has been reported to be critical, is one of the key mechanisms in EOC progression (4,6). Furthermore, the resistance to hypoxic-stress is essential for the induction of EMT and peritoneal dissemination (7,36). In the current study, it was demonstrated for the first time, to the best of our knowledge, that 4.1N may inhibit hypoxia-induced EMT of EOC cells at least partly via the regulation of HIF-1α.

Previous studies have indicated that the increased expression and activation of HIF are closely associated with cancer progression and poor prognosis of patients (37-39). HIF-1α has been previously reported to be correlated with the migration and invasion of EOC cells and has been demonstrated to be an important prognostic marker of patients with EOC (40-42). The results of the current study indicated that 4.1N may inhibit the nuclear accumulation of HIF-1α and suppress its expression.

Hypoxia-induced HIF activation is associated with a concomitant loss of E-cadherin (43), one crucial feature of EMT resulting in cancer metastasis and drug resistance. Imai *et al* (7) reported that immunolocalization of nuclear HIF-1α was correlated with the loss of E-cadherin in EOC cells. In the current study, the results indicate that absence of 4.1N may aggravate hypoxia-induced E-cadherin loss. It was observed that even under normoxic conditions, E-cadherin was additionally downregulated due to knockdown of 4.1N, which implied the potential interaction of 4.1N and E-cadherin. Additionally, the upregulation of hypoxia-induced N-cadherin expression was inhibited by 4.1N, which further suggested the role of 4.1N in impeding hypoxia-induced EMT. The data of the current study appears to be in support of an E/N-cadherin switch during EMT (44).

Several transcriptional factors have been demonstrated to be involved in EMT during tumor development. In the current study, Snail, Twist and LOX (18) were investigated, which are important molecules interacting with HIF-1α under hypoxic conditions (40). Snail and Twist have been demonstrated to induce EMT by repressing E-cadherin expression (45,46) through hypoxia signaling and the classical phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathways (31,47,48). In addition, the LOX family has multiple roles in tumor progression including altering extracellular matrix components (49) and regulating Snail (50). In EOC samples, LOX has been observed to be significantly associated with advanced clinical stages and metastasis (40). In the current study, a negative regulation of Twist, Snail and LOX by 4.1N further suggested a role of 4.1N in suppressing hypoxia-induced EMT.

According to the present data, an outline of the molecular pathway, which 4.1N is involved in during the hypoxia-induced EOC progression can be provided. On one hand, the promising hypoxia signalling pathway, HIF-LOX-Snail-E-cadherin (15) may assist in explaining the mechanisms by which 4.1N suppresses hypoxia-induced EMT. It was demonstrated that 4.1N regulated HIF, leading to the low expression of LOX and Snail and upregulation of E-cadherin. By contrast, it is also possible that 4.1N may affect the hypoxia-induced expression of LOX via association with PI3K. LOX-HIF-1α mutual regulation activated the AKT pathway in epithelial EOC (38) and it was previously reported that LOX activated the PI3K/Akt signaling pathway to upregulate HIF-1α protein synthesis (49). In addition, the association of 4.1N and PI3K was previoulsy demonstrated. Firstly, 4.1N was demonstrated to regulate PI3K activity via interactions with the PI3K (50). Secondly, our previous proteomic analysis indicated that 4.1N may upregulate the expression of inositol polyphosphate 5-phosphatase, an enzyme associated with PI3K. Therefore, 4.1N may deregulate the activity of PI3K and subsequently inhibit the expression of HIF-1α expression and Akt activity. LOX-HIF-1α mutual regulation was demonstrated to activate the Akt pathway in epithelial EOC cells (40). In addition, Pez *et al* (51) reported that LOX was able to activate the PI3K/Akt signaling pathway to upregulate HIF-1α protein synthesis and Ye *et al* (52) reported that 4.1N regulated PI3K activity via interactions with the PI3K enhancer. In addition, proteome analysis (Zhang *et al*, unpublished data) indicated that 4.1N may upregulate the expression of inositol polyphosphate 5-phosphatase, an enzyme associated with PI3K. It is notable that inositol polyphosphate 5-phosphatases may lead to apoptotic cell death (53), and share a potential role in tumor progression. Combining these two hypotheses, the predictive model presented in Fig. 4 is proposed. However, further studies are required to clarify the specific signaling networks.

In summary, the results of the present study indicate that 4.1N is an important regulator for hypoxia-induced EOC progression. 4.1N may be a potential tumor suppressor and a therapeutic target for patients with EOC.

Acknowledgements

The current study was supported by the National Natural Science Foundation of China (grant no. 81472430).

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