

## ALKBH3 Contributes to Survival and Angiogenesis of Human Urothelial Carcinoma Cells through NADPH Oxidase and Tweak/Fn14/VEGF Signals

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### Abstract

**Purpose:** The role and function of a novel human AlkB homologue, ALKBH3, in human urothelial carcinoma development were examined.

**Experimental design:** Biologic roles of ALKBH3 were examined by gene silencing analysis using *in vitro* and *in vivo* siRNA transfection. Immunohistochemical analyses of ALKBH3 and the related molecules using human bladder cancer samples were conducted to estimate the association with clinicopathologic or prognostic parameters.

**Results:** ALKBH3 knockdown induced cell cycle arrest at the G1 phase through downregulation of NAD(P)H oxidase-2 (NOX-2)-mediated generation of reactive oxygen species (ROS). ALKBH3 knockdown reduced VEGF expression by reducing expression of tumor necrosis factor-like weak inducer of apoptosis (Tweak) and its receptor, fibroblast growth factor-inducible 14 (Fn14). Silencing of ALKBH3 or Tweak significantly suppressed invasion and angiogenesis of urothelial carcinoma *in vivo* as assessed both by a chorioallantoic membrane assay and in an orthotopic mouse model. Interestingly, not only urothelial carcinoma cells but also vascular endothelial cells within cancer foci expressed Fn14, which was strongly reduced by ALKBH3 and Tweak knockdown *in vivo*, suggesting that ALKBH3-dependent expression of Tweak stabilizes Fn14. Immunohistochemical examination showed high expression of ALKBH3, Tweak, and Fn14 in urothelial carcinoma, especially in high-grade, superficially, and deeply invasive carcinomas; moreover, Fn14-positive vessel counts within cancer foci were increased in invasive phenotypes.

**Conclusions:** ALKBH3 contributes to development of urothelial carcinomas by accelerating their survival, angiogenesis, and invasion through NOX-2-ROS and Tweak/Fn14-VEGF signals. *Clin Cancer Res*; 18(19); 5247–55. ©2012 AACR.

### Introduction

In *Escherichia coli*, the AlkB protein, a repair enzyme of methylation-induced DNA damage, is implicated in preventing carcinogenesis (1, 2). Human homologues of the AlkB family include the genes ALKBH1-8 (3). We have tried to identify the functional roles of ALKBH members in the progression of several types of human cancer. For example, ALKBH8 is mainly involved in resistance to cell death in human urothelial carcinoma cells, resulting in

human bladder cancer development (4). ALKBH3 plays an important role in human pulmonary adenocarcinoma or prostatic adenocarcinoma cell survival and may be a therapeutic target (5, 6). These reports show that ALKBH members are involved in a variety of biologic functions, including DNA repair, and may be of high-therapeutic value.

Reactive oxygen species (ROS) are implicated in both stimulation and inhibition of cell proliferation, apoptosis, and cell senescence (7, 8). ROS trigger genetic programs associated with oncogene-mediated transformation (9, 10). NADPH oxidase (NOX) is one of the major sources for cellular ROS (11, 12). NOX enzymes are the structural homologues of phagocytic NOX (gp91phox/NOX2) and consist of both single (NOX-1–NOX-5) and dual oxidases (DuOX1 and DuOX2). There is mounting evidence implicating low levels of ROS generated by NOX enzymes as mediators in inflammation, apoptosis, cell growth, and angiogenesis in various types of human cancers. A typical TNF superfamily member, TNF-like weak inducer of apoptosis (Tweak), is expressed by not only hematopoietic lineage cells but also endothelial cells and cancer cells, and is implicated in the pathogenesis of

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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### Translational Relevance

High-grade and muscle invasive cancers progress to local and distant metastases despite radical cystectomy or systemic chemotherapy, but the target molecule remains elucidated. *In vitro*, *in vivo*, and pathologic examinations clearly showed that ALKBH3 plays an important role in cancer cell survival, angiogenesis, and invasion in human urothelial carcinomas through NOX2-ROS signaling in urothelial carcinoma cells, which may be involved in early stages of cancer development, and by inducing Tweak/Fn14 and dependent VEGF induction in cancer cells. In addition, ALKBH3-mediated expression of Tweak stabilizes Fn14 in both cancer cells and endothelial cells of cancer microvessels. Thus, ALKBH3 can be a new molecular target of therapy for urinary bladder cancer, especially a highly advanced phenotype.

cancer. Production of Tweak in the tumor microenvironment can stimulate angiogenesis, which is crucial for primary tumor growth and metastasis (13, 14). Ho and colleagues (15) showed HEK293 cells overexpress Tweak form large, highly vascularized tumors when injected subcutaneously into mice. The receptor for Tweak, fibroblast growth factor-inducible 14 (Fn14), is a member of the TNF superfamily of receptors and is well known to be closely associated with cell growth, adhesion, and migration in some cancers, including hepatocellular carcinomas and pancreatic cancer (16–18). Moreover, overexpression of Fn14 was found to promote androgen-independent prostate cancer progression through matrix metalloproteinase 9 (MMP-9) and correlates with poor treatment outcome (19). Fn14 contains a binding site for members of the TNFR-associated factor family that activates nuclear factor- $\kappa$ B (20). It would thus seem that NOX and downstream targets, including ROS and Fn14-related signals, are necessary for cancer development, and would therefore make attractive targets for therapeutic intervention in cancer development. However, how NOX-ROS and Tweak/Fn14, and the upstream molecules that regulate these signals, enhance bladder cancer development has not been fully elucidated.

In the present study, we show that ALKBH3 is an upstream molecule of NOX2 and Tweak induction. We also show that NOX2 mediates cell survival through accelerating the cell cycle, whereas Tweak stabilizes Fn14 expression and executes Fn14-mediated angiogenesis, including VEGF induction. It is of great interest that Tweak production from cancer cells is closely associated with Fn14 induction of endothelial cells within the cancer foci, which is mainly involved in development of invasive urothelial carcinomas (pT<sub>1</sub>) and high-grade carcinoma *in situ* (CIS). On the basis of these findings, we suggest that ALKBH3 and dependent signals may serve as useful target molecules for human urinary bladder cancer therapy.

### Materials and Methods

#### Cell culture, plasmids, and chemicals

The human urothelial carcinoma cell lines, UMUC2 and UMUC3, were purchased from American Type Culture Collection and cultured in RPMI supplemented with 10% FBS. Antibodies for actin, VEGF, Fn14 (which can react with Fn14 of human and mouse origins) were from Santa Cruz Biotech. Inc.; antibodies for NOX2 and Tweak were from Abcam. Alexa-Flour goat antirabbit immunoglobulin G (IgG) and antimouse IgG were from Invitrogen.

#### Preparation of antisera

Anti-ALBH3 antisera were prepared as described previously against a synthetic perchloric acid 1 peptide (amino acids 64–76) as the antigen (5). After a 0.5 mg aliquot of peptides was emulsified and injected into rabbits, blood was collected at 2-week intervals. The relative activity of antisera against the synthetic peptide was tested by ELISA.

#### Transfection of human ALKBH3 siRNA *in vitro*

The cells were seeded at  $10^6$  cells per well in 6-cm plates, then transfected with 100 nmol/L control RNA (Santa Cruz), siRNAs of human ALKBH3, Tweak or Fn14 [horseradish peroxidase (HRP)-validated siRNA S102663892 for ALKBH3; S100056126 for Tweak; S103047621 for Fn14] from Qiagen or NOX2 from Santa Cruz using lipofectamine (Invitrogen) in accordance with the manufacturer's protocol. After cultivation for the indicated time, the samples were removed and homogenized, then the lysates were used for immunoblotting/immunohistochemistry (IHC).

#### Preparation of cell lysates and Western blot analysis

We resolved the cell lysates in SDS-polyacrylamide gels and transferred them to polyvinylidene difluoride membranes (Millipore, Ltd.), which were then blocked in 5% skim milk at room temperature for 1 hour. We incubated the membranes with the indicated primary antibody for 1 hour, and then incubated with HRP-conjugated anti-mouse or -rabbit IgG (Amersham Pharmacia Biotech). We detected peroxidase activity on X-ray films using an enhanced chemiluminescence detection system.

#### Identification and measurement of ROS

We assessed the production of intracellular superoxide anion ( $O_2^-$ ) and  $H_2O_2$  with dihydroethidium (DHE; Wako) and 5,6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-DCF-DA; Wako), respectively. Cells were incubated with a 5  $\mu$ mol/L DHE or DCF-DA solution for 30 minutes at 37°C. Excess DHE or DCF-DA was washed, then superoxide anion ( $O_2^-$ ) or  $H_2O_2$  formation in the cells was visualized using a Leica CTR6000 photomicroscope and quantitatively measured by flow cytometry.

#### Cell cycle analysis

We carried out cell cycle analyses by flow cytometry as previously described (4). All experiments were done at least thrice in duplicate.

### Chorioallantoic membrane assay

KU7-GFP Cells ( $10^6$ ) were seeded on the chorioallantoic membranes (CAM) of 11 days old chick embryos. After a 24-hour cultivation, we added control RNA or 10  $\mu\text{mol/L}$  siRNA for the indicated gene combined with atelocollagen (Atelogene, Koken Co., Ltd.) according to the manufacturer's protocol. Five days postimplantation, CAM samples were collected and fixed, then stained with anti-human cytokeratin antibody (CKAE1/AE3; DAKO) to allow invading cytokeratin-positive cells to be quantified. The depth of invasion from the CAM surface was defined as the leading front of 3 or more invading cells in 5 randomly selected fields. We assessed angiogenesis as the number of visible blood vessel branch points within a defined area of the sample (4, 5). The remaining samples are served for Western blotting. At least 3 CAMs were used for each experiment.

### Cell proliferation assay

After stimulation with various reagents for a given period, cells were treated by MTS reagent (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphonyl) -2H-tetrazolium, inner salt; Promega). After a 3-hour incubation period, optical absorbance at 490 nm was measured using a microplate reader. All experiments were carried out in triplicate.

### Orthotopic tumor implantation and intravesical treatment

The method used for the orthotopic instillation of tumor cells has been described (4); briefly, we anesthetized 8-week-old female athymic nude mice and inserted a 24-gauge catheter transurethrally into the urinary bladder. Then,  $1 \times 10^7$  GFP-tagged KU7 cells suspended in 100  $\mu\text{L}$  medium were instilled into the bladder lumen and the urethra was ligated for 2 to 3 hours. Fourteen days after the cell instillation, we transurethrally injected either control RNA ( $n = 5$ ) or 10  $\mu\text{mol/L}$  of the ALKBH3 or Tweak siRNA + atelocollagen mixture ( $n = 5$ ) previously described (Koken Co., Ltd.) into the urinary bladder (retain the RNA for 1 hour).

### In vivo fluorescence and image analyses

Twenty-eight days after the instillation of tumor cells, digital images were captured under both fluorescent and incandescent light. Image analysis was done with Image J public domain software available through the NIH. All images were spatially calibrated for area measurements. The mice were then sacrificed, and the bladders were excised and fixed for histologic examination by hematoxylin and eosin stain (H&E) and IHC.

### Tissue samples and IHC

We obtained specimens of human urothelial carcinomas ( $n = 93$ ) from patients undergoing transurethral resection or radical cystectomy, without previous radiation or chemotherapy, at Nara Medical University Hospital (Table 1). Clinicopathologic data of the present cases were reviewed

**Table 1.** Characterization of urothelial carcinomas

<b>Mean age (y)</b>	<b>72.2 (44–96)</b>
<b>Gender</b>	<b>70:23 (M:F)</b>
<b>Pathologic stage</b>	
pTis	14 (15%)
pTa	29 (31%)
pT1	27 (29%)
pT2	23 (25%)
<b>Grade</b>	
Low grade	51 (55%)
High grade	42 (45%)

by 2 urologic pathologists (K. Shimada and N. Konishi, Department of Pathology, Nara Medical University Hospital) and summarized in Supplementary Table S1. Normal or atypical urothelial tissue samples found in patients with or without severe cystitis were from autopsy cases ( $n = 17$  for each). The current research was approved by Institutional Research Board of Nara Medical University and informed consent was obtained from all patients. Before the collection of specimens, we reevaluated tumor stage and grade at initial diagnosis. We followed the same tissue fixation and processing procedure as described in a previous report (21, 22). Percentages of cells positive for ALKBH3 were expressed per 1,000 cells examined.

### Statistical analysis

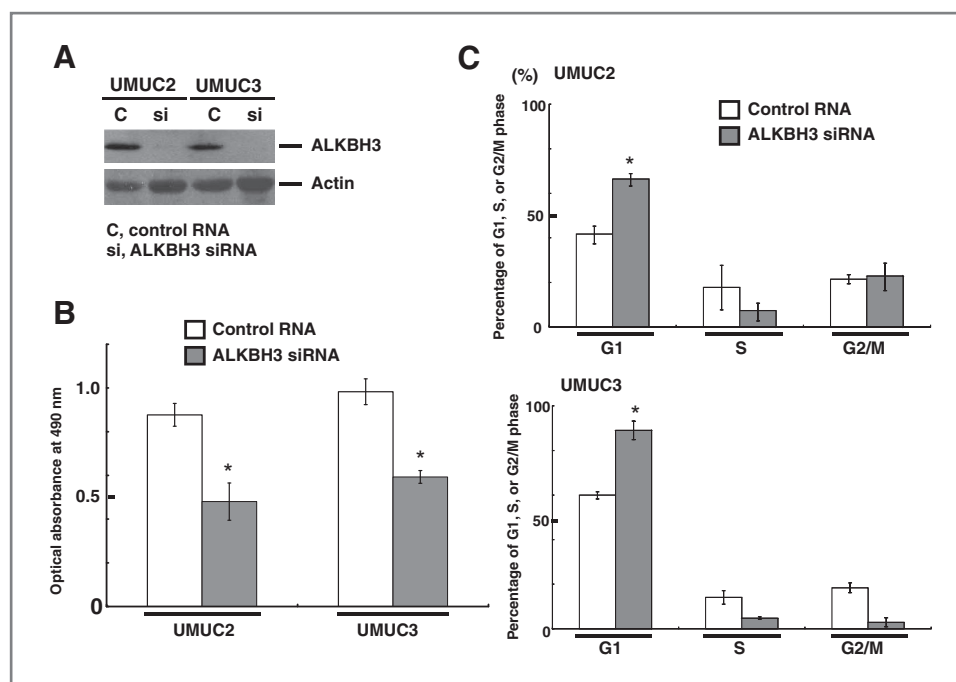
Differences in the means of continuous variables were analyzed using ANOVA or nonparametric (Mann–Whitney, Kruskal–Wallis) tests.

All experiments were analyzed using a 1-way ANOVA and the Tukey *post hoc* test. A 2-tail Students *t* test was used to compare two data points. Graphical results represent mean  $\pm$  S.E. Results were deemed statistically significant at the level of  $P < 0.05$ , and experiments were done with  $n \geq 3$ .

### Results

#### ALKBH3 knockdown induces cell cycle arrest through downregulation of NOX2 expression in urothelial carcinoma cells

Protein expression was strongly reduced in both the human urothelial carcinoma cell lines UMUC2 and UMUC3, when transfected with 100 nmol/L of ALKBH3 siRNA for up to 72 hours (Fig. 1A). As shown in Fig. 1B and C, ALKBH3 gene silencing resulted in cell cycle arrest at the G1 phase, and cancer cell growth was significantly suppressed. Given that ALKBH8, an ALKBH family members, ALKBH8, contribute to ROS generation through NOX1 in human urothelial carcinoma cells (4), we examined whether ROS levels in cancer cells were affected by ALKBH3 gene expression. Figure 2A and B illustrate that intracellular generation of superoxide anion and hydrogen peroxide were strongly depressed by ALKBH3 knockdown in UMUC3 and UMUC2 cells as measured by flow cytometric



**Figure 1.** Downregulation of ALKBH3 induced cell cycle arrest in human urothelial carcinoma cells. A, UMUC2 and UMUC3 cells were transfected with 100 nmol/L of ALKBH3 siRNA or control RNA. Expression of ALKBH3 protein was examined by Western blotting (densitometric value control:siRNA: ALKBH3, 1:0.12 and 1:0.21 in UMUC2 and UMUC3 cells, respectively). B and C, MTS assay and flow cytometric analysis using propidium iodide were conducted. Actin and GAPDH were used as internal controls throughout whole experiments. Bars, SE; columns, mean. \*, statistically different from the value of control at  $P < 0.01$ .

analysis. Western blot showed that ALKBH3 gene silencing decreased NOX2 expression (Fig. 2B), but did not affect expression of other related molecules such as NOX1, NOX3, NOX4, NOX5, Duox1, or Duox2 (data not shown). In addition, the number of cells in the G1 fraction was increased by NOX2 downregulation in both cell lines (Fig. 2C). These results indicate that ALKBH3 and downstream NOX2-ROS is closely associated with urothelial carcinoma cell growth.

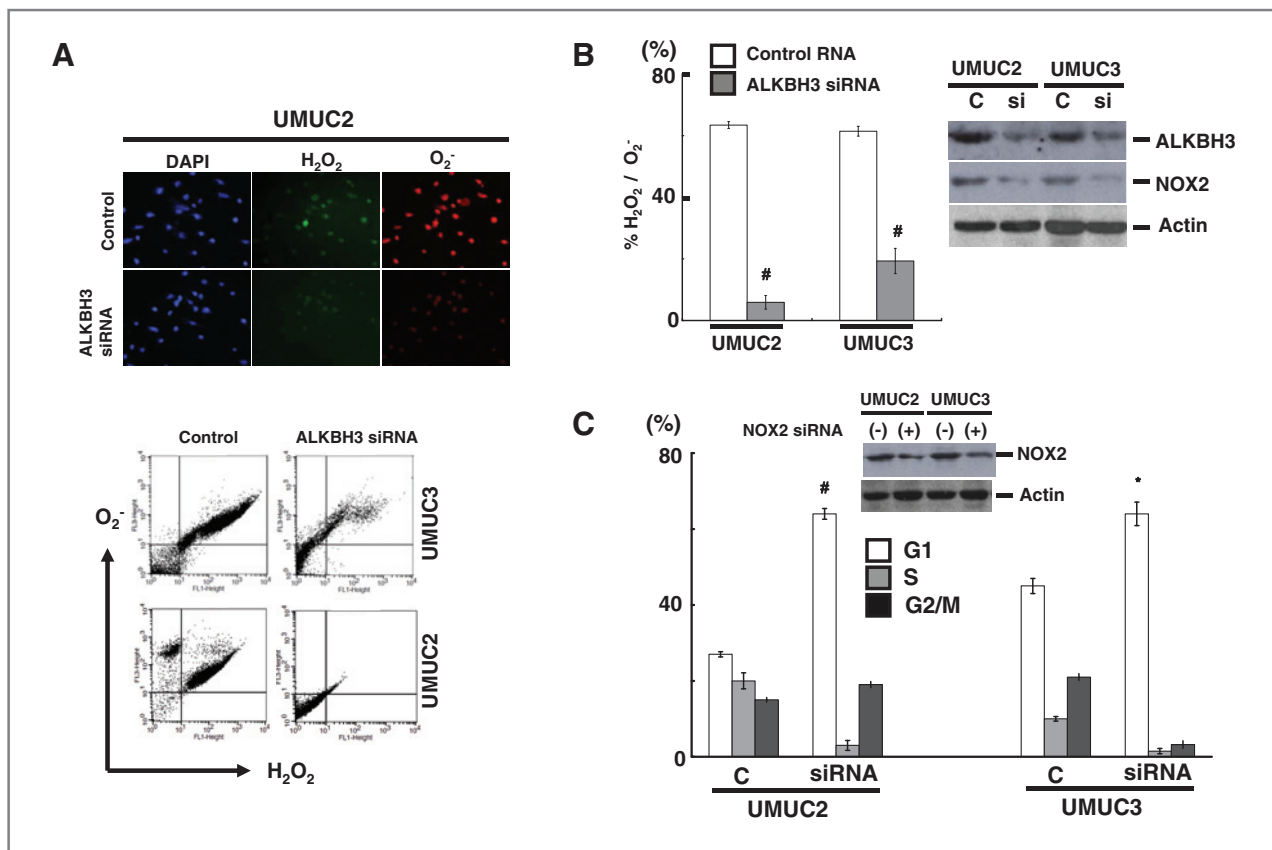
#### Invasion and angiogenesis are suppressed by ALKBH3 gene silencing in urothelial carcinoma cells

Results from a CAM assay revealed significant suppression of angiogenesis and depth of invasion by *in vivo* transfection of ALKBH3 siRNA into KU7 cells (Fig. 3). Because the efficacy of suppression *in vivo* was much more prominent than the growth inhibitory effect induced by ALKBH3 downregulation *in vitro*, neovascularization may be one of the primary mechanisms that enhance cancer progression by ALKBH3. We then examined several molecules involved in the process of angiogenesis and found that Tweak, Fn14, and VEGF were strongly affected by ALKBH3 gene silencing (Fig. 3B). However, other proteins such as basic fibroblast growth factor and platelet-derived growth factor were not affected (data not shown). Tweak knockdown strongly reduced expression of Tweak receptors, Fn14, and VEGF. In addition, expression of VEGF was reduced by Fn14 gene silencing (Fig. 3C). NOX2 expression was not affected by Tweak/Fn14 gene silencing. In contrast, the knockdown of NOX2 had no significant effects on these molecules, except for a slight reduction in VEGF expression. Results from the CAM assay showed that invasion depth was predominantly

inhibited by Fn14 gene silencing rather than NOX2 downregulation (Fig. 3D). These findings strongly implicate Tweak/Fn14 as a major upstream factor of VEGF induction and angiogenesis, which plays an important role in ALKBH3-mediated invasion in human urothelial carcinoma cells.

#### *In vivo* growth of urothelial carcinoma is suppressed by ALKBH3 siRNA transfection in the orthotopic implant mouse model

We have recently established a method that uses GFP image analysis to quickly measure tumor growth and treatment response, which offers many advantages over slower histologic methods that use KU7 cell lines stably overexpressing GFP (4). As shown in Fig. 4A top panel, ALKBH3 downregulation by *in vivo* transfection of siRNA, combined with atelocollagen, resulted in an approximately 6-fold decrease in tumor area. Expression of ALKBH3 downstream molecules, NOX2, Tweak, Fn14, and VEGF, was significantly reduced by ALKBH3 knockdown (Fig. 4A, bottom panel). Conventional histologic evaluation revealed similar results: large tumors with cancer cells deeply infiltrating into the bladder wall were observed in control mice, whereas only a few foci of cancer cells were observed in mice treated by ALKBH3 gene silencing. The number of vessels within cancer foci was also suppressed as assessed by counting of CD31-positive vessels. From IHC using anti-Fn14 antibody reacting to tissues of human and mouse origins, almost all vascular endothelial cells within cancer foci were shown to express Fn14 in control mice, whereas in mice receiving ALKBH3 siRNA, the number of vascular endothelial cells expressing Fn14 was significantly lower (Fig. 4B).



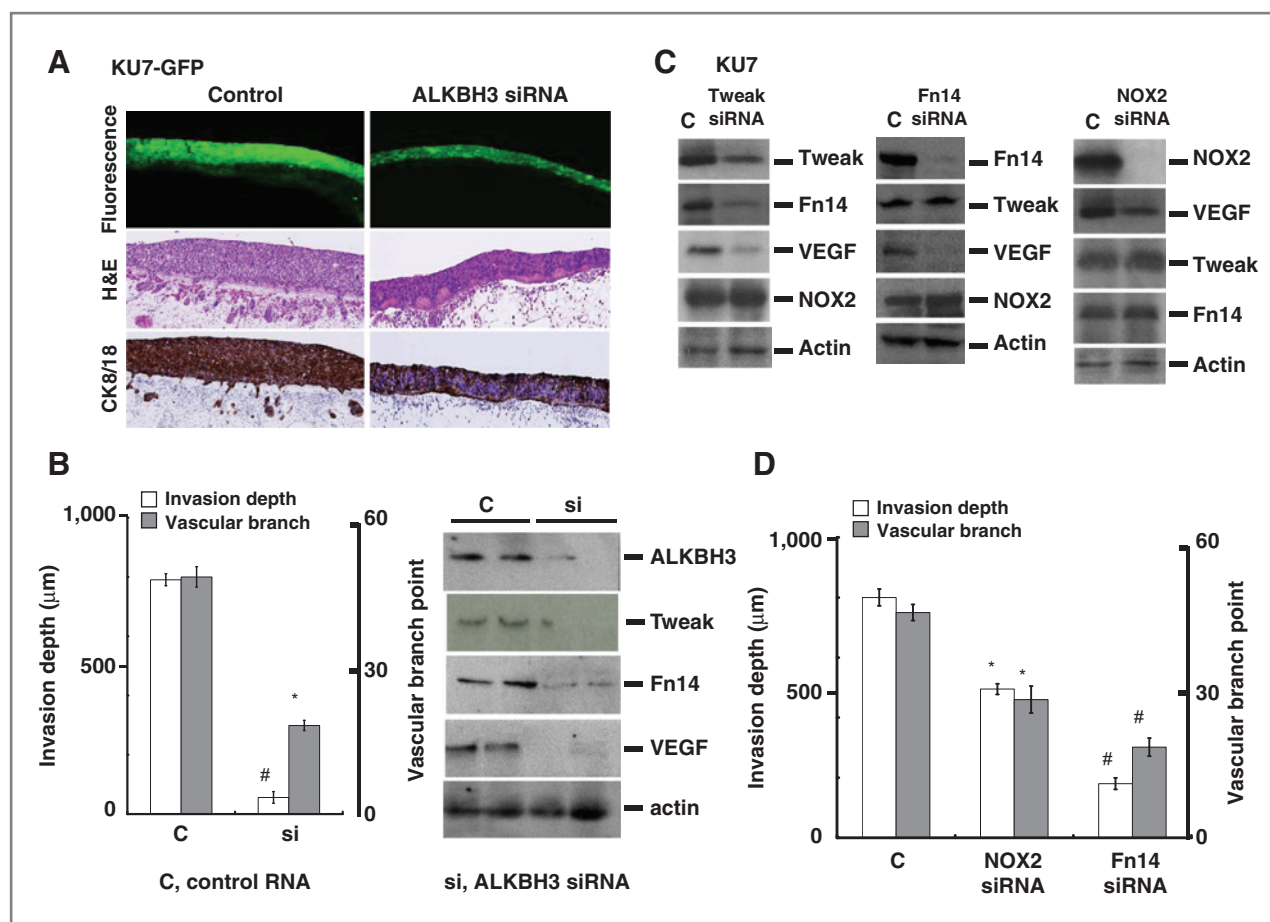
**Figure 2.** The ALKBH3- and NOX2-mediated generation of ROS in human urothelial carcinoma cells. **A**, UMUC2 cells were treated by ROS detecting fluorescence reagents, CM-DCF-DA and DHE, and ROS generation was assessed by fluorescence microscopy (top). UMUC2 and UMUC3 cells were transfected by ALKBH3 siRNA at 100 nmol/L and control RNA. After 72 hours inoculation, cells were treated with CM-DCFDA and DHE, then intracellular ROS levels were analyzed by flow cytometer (bottom). **B**, generation of hydrogen peroxide and oxygen radicals was quantitatively analyzed by flow cytometer (left) and Western blotting using anti-ALKBH3 or NOX2 antibody was conducted (right; densitometric value control:siRNA: ALKBH3, 1:0.23 and 1:0.31; NOX2, 1:0.37 and 1:0.20 in UMUC2 and UMUC3 cells, respectively). **C**, UMUC2 and UMUC3 cells were transfected by NOX2 siRNA at 100 nmol/L and control RNA. After 72 hours inoculation, flow cytometric analysis using propidium iodide or Western blotting using anti-NOX2 antibody was conducted (densitometric value control:siRNA: NOX2, 1:0.41 and 1:0.33 in UMUC2 and UMUC3 cells, respectively). Bars, SE; columns, mean; C, control RNA; si, ALKBH3 siRNA. \* and #, statistically different from the value of control at  $P < 0.01$  and  $P < 0.001$ , respectively.

### Tweak stabilizes Fn14 expression in endothelial cells of tumor vessels in the orthotopic implant mouse model

*In vivo* knockdown of Tweak in the orthotopic implantation model strongly inhibited tumor growth, to an extent similar to that observed for ALKBH3 downregulation. Tweak knockdown also reduced expression of both Fn14 and VEGF in cancer foci as assessed by Western blotting (Supplementary Fig. S1A). NOX2 expression was not modified by Tweak gene silencing (data not shown). Immunohistochemical analysis showed downregulation of Fn14 expression in not only cancer cells but also endothelial cells of tumor microvessels (Supplementary Fig. S1B). Moreover, tumor vessel density was significantly decreased by Tweak siRNA transfection with atelocollagen. Nearly all endothelial cells within cancer foci expressed Fn14, which was strongly depressed by Tweak knockdown *in vivo*. These data indicate that Fn14 expression, controlled by ALKBH3/Tweak, is essential for effective angiogenesis and progression of urothelial carcinomas.

### Immunoprofile of ALKBH3 and downstream molecules in human urothelial carcinoma of the urinary bladder

We conducted immunohistochemical analysis of ALKBH3 and the downstream molecules, Tweak/Fn14 and NOX2, in human bladder cancer specimens to examine potential correlations between their expression, tumor grade, and invasiveness. The percentages of cells immunopositive for ALKBH3 were much higher in high-grade urothelial carcinomas, including CIS, with both minimal and wide invasion (pT<sub>1</sub>) than in low-grade lesions with a noninvasive phenotype (pT<sub>a</sub>; Supplementary Fig. S2). Immunohistochemical analyses of Tweak and Fn14 showed the same results (Supplementary Fig. S3). Similarly, NOX2 was overexpressed in urothelial carcinoma cells, but exhibited very low expression in normal urothelium; however, the percentages for immunopositive cells were not associated with histologic grade or stage (Supplementary Fig. S4). Percentages of positive cells for ALKBH3 at initial diagnosis of pT<sub>a</sub>/pT<sub>1</sub> cancers ( $n = 47$ ) was statistically associated with multiplicity, tumor size (more or less than 3 cm), and the



**Figure 3.** Silencing ALKBH3 reduced angiogenesis and invasion of urothelial carcinoma cells *in vivo* in the CAM assay. KU7/GFP cells were seeded on top of the CAMs of 11 day old chicks and atelocollagen-control RNA or atelocollagen-ALKBH3 siRNA mixture was added, and then the cells were incubated for 5 days after implantation. The fluorescence microscopic finding, histologic data of H&E, or IHC by cytokeratin AE1/AE3 are shown in (A). Invasion depth and angiogenesis were analyzed (B, left), and cancer samples were resected and lysed, and expression of ALKBH3, Tweak, Fn14, or VEGF was examined in Western blotting (B, right; densitometric value control:siRNA: ALKBH3, 1:0.06 ~0.14; Tweak, 1:0.07 ~ 0.10; Fn14, 1:0.18 ~ 0.20; VEGF, 1:0.05 ~ 0.13). C, cells were transfected by Tweak, Fn14, or NOX2 siRNA at 100 nmol/L and control RNA *in vitro*. After 72 hours inoculation, expression of Tweak, Fn14, VEGF, or NOX2 was analyzed by Western blotting (densitometric value control:Tweak siRNA: Tweak, 1:0.41; Fn14, 1:0.25; VEGF, 1:0.18; NOX2, 1:1.18; densitometric value control:Fn14 siRNA: Fn14, 1:0.11; Tweak, 1:1.08; VEGF, 1:0.10; NOX2, 1:1.14; densitometric value control:NOX2 siRNA: NOX2, 1:0.07; VEGF, 1:0.64; Tweak, 1:1.1; Fn14, 1:1.06). D, invasion depth and angiogenesis were analyzed by the CAM assay using KU7 cells receiving control, or NOX2 (or Fn14) siRNA in the presence of atelocollagen. Bars, SE; columns, mean. \* and #, statistically different from the value of control at  $P < 0.01$  and  $P < 0.001$ , respectively.

time of recurrence within 5 years, suggesting that immunoreactivity for ALKBH3 may be a useful marker for predicting malignant potential of urothelial carcinomas (Supplementary Fig. S5).

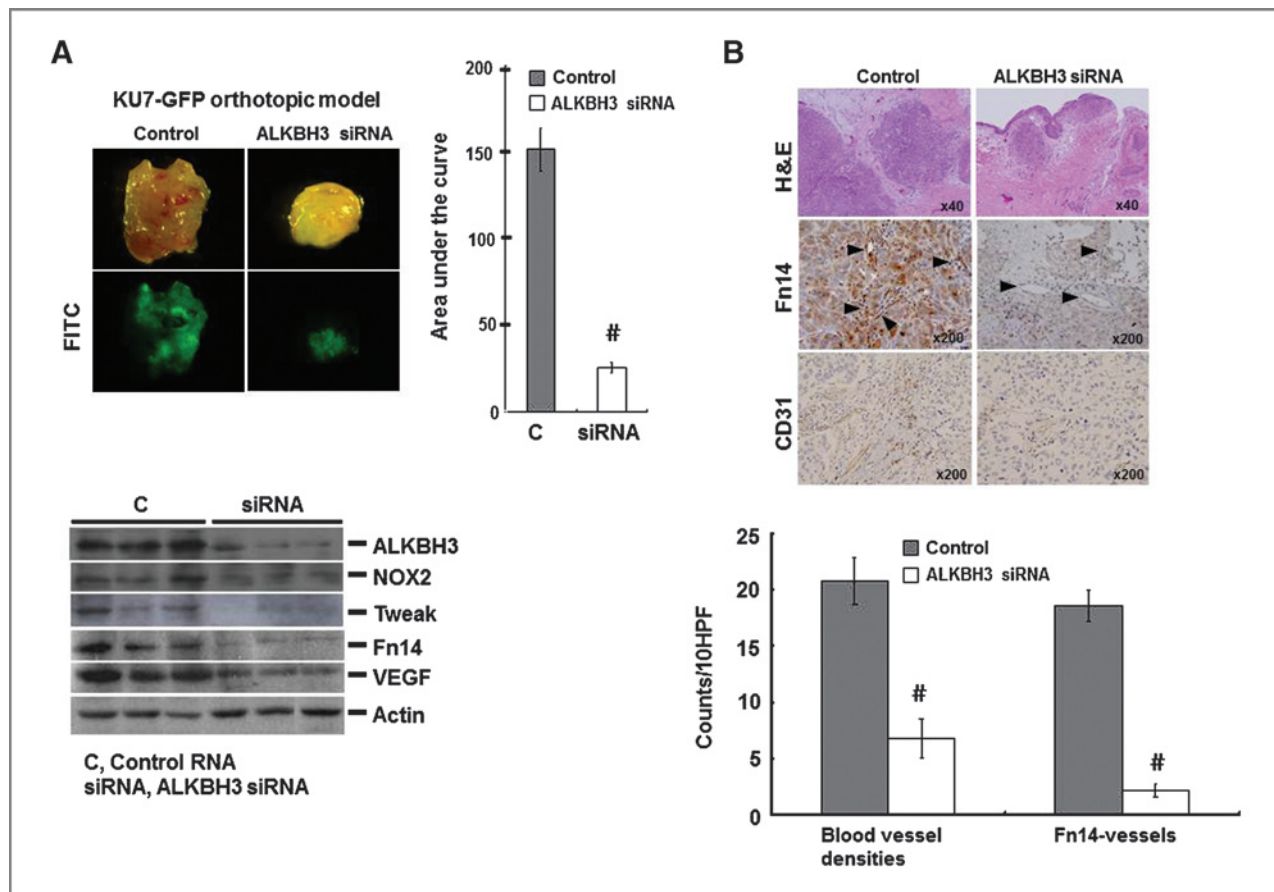
#### Tweak/Fn14 expression in endothelial cells of microvessels within invasive/high-grade urothelial carcinoma foci

We examined the immunoprofile of Tweak and Fn14 proteins on microvessels within cancer foci by Tweak/CD31 and Fn14/CD31 double-staining methods. Figure 5A shows Tweak/Fn14 was highly expressed in endothelial cells of microvessels close to the border of cancer foci. The percentages of endothelial cells immunopositive for Tweak and Fn14 were significantly higher in superficial and deeply invasive urothelial carcinomas (pT<sub>1</sub>, pT<sub>2</sub>) as well as high-

grade CIS than in normal urothelium or noninvasive/low-grade urothelial carcinomas (pT<sub>a</sub>; Fig. 5B).

#### Discussion

We show here for the first time that ALKBH3 plays important roles in the progression of urothelial carcinoma cells through NOX2-dependent ROS generation and Tweak/Fn14-VEGF axes. *In vitro* experiments using human urothelial carcinoma cell lines clearly showed that growth suppression by ALKBH3 knockdown is mainly mediated by G1 cell cycle arrest through downregulation of NOX2-ROS generation. Thus, the function of NOX2 is required for urothelial carcinoma cell survival by ALKBH3. Recently, Kumar and colleagues reported that inhibition of ROS generation by NOX inhibitors suppressed invasion capacity by decreasing MMP-9 in prostate cancer cells. However, in

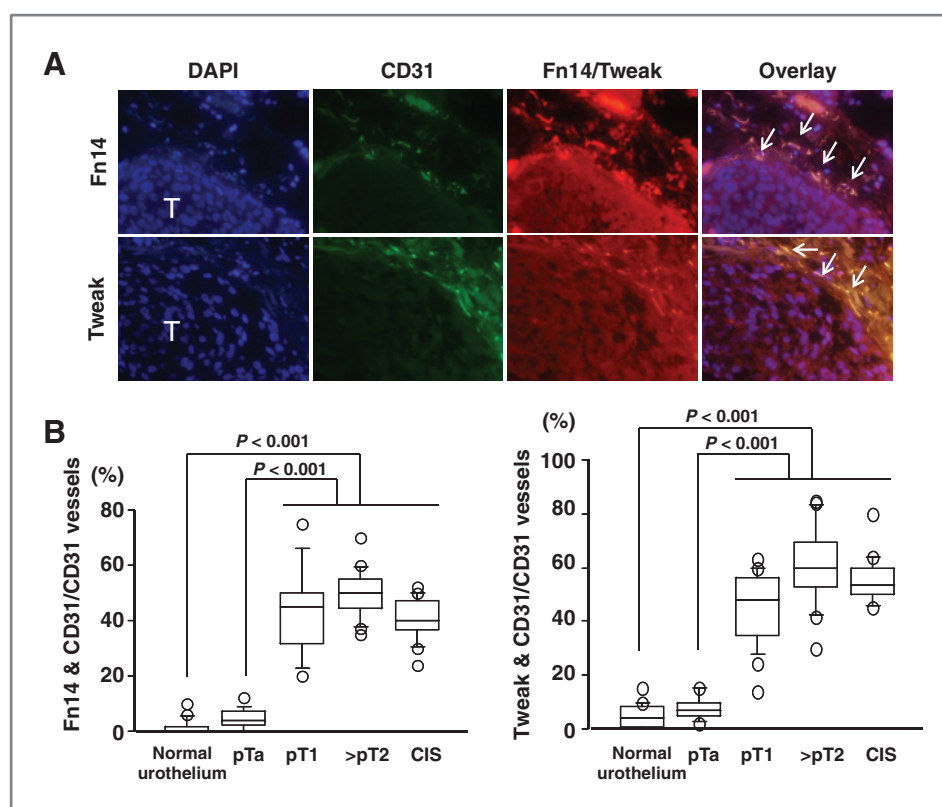


**Figure 4.** Intravesical injection of ALKBH3 siRNA inhibits tumor growth *in vivo* in the mouse orthotopic bladder cancer implant model. **A**, 14 days after KU7/GFP cells were transplanted into the mouse bladder, atelocollagen with either ALKBH3 siRNA or control RNA was transurethraly instilled into the bladder lumen. Bladders were resected 14 days postinstillation, *in situ* images were captured under fluorescent light, and tumor area was measured by GFP expression (top). Bladder cancer samples were resected and lysed, and expression of ALKBH3, NOX2, Tweak, Fn14, or VEGF was examined in Western blotting (bottom; densitometric value control:ALKBH3 siRNA: ALKBH3, 1:0.15 ~ 0.34; NOX2, 1:0.10 ~ 0.38; Tweak, 1:0.21 ~ 0.45; Fn14, 1:0.16 ~ 0.21; VEGF, 1:0.18 ~ 0.25). **B**, the microscopic data of H&E or IHC by Fn14 or CD31 are shown in the top. Number of blood vessels (positive for CD31) or Fn14 positive blood vessels per 10 high-power fields within cancer foci was counted in lower panel. Bars, SE; columns, mean. #, statistically different from the value of control at  $P < 0.001$ .

our study, expression and activity of MMPs were not significantly modified even though ROS was similarly suppressed, and invasion capacity and angiogenesis were not inhibited by NOX2 knockdown to the same extent as ALKBH3 gene silencing. Moreover, NOX2 was found to be highly expressed in cancer cells, but the positive percentages were not statistically correlated to histologic grade and stage of urinary bladder cancer. Thus, NOX-ROS signaling appears to have no significant effects on cancer angiogenesis and invasiveness in human bladder cancer. However, several lines of evidence suggest an important role for NOX in tumor angiogenesis; for example, ROS generation through NOX4 contributes to induction of angiogenesis through upregulation of VEGF and hypoxia-inducible factor-1 $\alpha$  in ovarian cancer cells (23, 24). Abid and colleagues (25) found that NOX activity is required for VEGF receptor-mediated cell signaling. This discrepancy may be due to the cell type- and/or isoform-specific action of NOX. With respect to the function of ROS in NOX signaling, a number

of conflicting reports have also accumulated. The overproduction of ROS contributes to cytotoxicity by extracellular stimuli such as anticancer drugs. Whereas the explosive production of ROS stimulated by cytotoxic drugs promotes cell death, low endogenous levels of ROS generated mainly by ALKBHs-NOXs have been linked to increased aggressiveness of cancer cells. Thus, a better understanding of the effects of differences in intracellular levels of ROS will be key to elucidating their complicated functions.

In contrast to NOX2, manipulation of Tweak and Fn14 did not significantly affect cytotoxicity *in vitro* (data not shown), but instead resulted in strong suppression of VEGF and angiogenesis examined by the CAM assay and orthotopic implantation model. In addition, we found that Tweak itself stabilized the receptor, Fn14, on both endothelial cells of tumor vessels and cancer cells *in vivo*. Interestingly, Donohue and colleagues (26) recently reported that VEGF could directly induce Fn14 expression of endothelial cells. Although their experiments were not specific to



**Figure 5.** Tweak/Fn14 overexpression on endothelial cells is associated with invasion of urothelial carcinomas. **A**, immunofluorescence by Tweak/Fn14 or CD31 was conducted. Merge of CD31 and Tweak or Fn14 immunostaining shows that Tweak or Fn14 was expressed in almost endothelial cells of microvessels at the margin of invasive cancer foci. **B**, percentages of double-positive cells for Tweak (or Fn14) and CD31 per all CD31 positive endothelial cells were shown.

cancer research, their findings suggest that positive feedback regulation of Tweak-/Fn14-mediated angiogenesis could be executed by downstream VEGF in urothelial carcinoma cells. In addition to VEGF, Tweak is well known to be an important angiogenetic factor; however, the correlation between Tweak/Fn14 and VEGF is not fully understood. Our present data are the first to show that Tweak successfully mediates tumor angiogenesis by means of direct and indirect actions through Fn14 stimulation and VEGF induction, respectively. With the involvement of ALKBH3, Tweak acts in concert with VEGF to stimulate a synergistic endothelial cell growth in human urothelial carcinoma cells. The fact that neovascularization was strongly inhibited by Tweak silencing *in vivo*, and VEGF was shown to be closely regulated by Tweak/Fn14 signals, suggests that the involvement of Tweak is more important than VEGF with respect to ALKBH3-mediated cancer progression. Another important finding of the present study was that suppression of invasion capacity by Tweak/Fn14 knockdown was much higher than that observed for NOX2 gene silencing, indicating that angiogenesis by Tweak/Fn14 is likely more important for bladder cancer invasion controlled by ALKBH3 than for cell cycle progression by NOX2. This finding is supported by 2 additional lines of evidence from previous reports, and the present study: (i) it has been shown that microvessel density is markedly increased in invasive urothelial carcinomas (27, 28); and (ii) we showed that ALKBH3 expression in cancer cells and Tweak/Fn14 expression on both cancer and microvascular endothelial cells within cancer foci were dramati-

cally increased in noninvasive urothelial carcinomas compared with invasive urothelial carcinomas, whereas NOX2 expression was not statistically correlated to pathologic stage. Unfortunately, we have no data to explain the function of NOX2 in invasive urothelial carcinoma cells or the biologic roles of Tweak/Fn14 in the low-grade/noninvasive phenotype, both of which warrant further investigation. It is well known that CIS frequently progresses to invasive urothelial carcinoma; however, the key mechanisms directing this process remain unclear. The present study showed Tweak/Fn14 was overexpressed in not only cancer cells but also in microvessels directly below the basement membrane of CIS; therefore, CIS cancer cells are likely prepared for invasion by a Tweak-/Fn14-dependent angiogenesis mechanism controlled by ALKBH3. Finally, our data revealed that ALKBH3 could be a useful marker for prediction of bladder cancer recurrence or multiplicity, and help direct appropriate postoperative examination or treatment regimens for cases of urothelial carcinomas with high expression of ALKBH3 at initial diagnosis.

In summary, ALKBH3 plays an important role in cancer cell growth, angiogenesis, and invasion in human urothelial carcinomas by enhancing the turnover of the cell cycle by NOX2-ROS signaling in urothelial carcinoma cells, which may be involved in early stages of cancer development, and by inducing Tweak/Fn14 and dependent VEGF induction in cancer cells. In addition, we found that ALKBH3 stabilizes Fn14 through Tweak in both cancer cells and endothelial cells of cancer microvessels (Supplementary Fig. S6). Thus,



ALKBH3 can initiate effective ang Tweak knockdown strongly reduced expression iogenesis through the manipulation of Tweak/Fn14 and VEGF, resulting in a highly advanced phenotype of urothelial carcinoma cells.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Authors' Contributions

**Conception and design:** K. Shimada, N. Konishi

**Development of methodology:** K. Shimada, K. Tsujikawa

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** K. Shimada, S. Anai, K. Fujimoto

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** K. Shimada, S. Anai, N. Konishi

**Writing, review, and/or revision of the manuscript:** K. Shimada, N. Konishi

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** T. Fujii, K. Fujimoto, N. Konishi

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