ALKBH3 contributes to survival and angiogenesis of human urothelial carcinoma cells through NADPH oxidase and Tweak/Fn14/VEGF signals

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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 pathological 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.

Abstract

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

Experimental design: Biological 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 performed to estimate the association with clinicopathological 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 vascular endothelial growth factor (VEGF) expression by
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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.
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 demonstrate that ALKBH members are involved in a variety of biological 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 tumor necrosis factor (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 cancer. Production of TWEAK in the tumor microenvironment can stimulate angiogenesis, which is crucial for primary tumor growth and metastasis (13, 14). Ho et al. (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 (TRAF) family that activates nuclear factor-κB (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 demonstrate 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 vascular endothelial growth factor (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 (≥ pT1) 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 (Manassas, VA) and cultured in RPMI supplemented with 10% fetal bovine serum. Antibodies for actin, Vascular endothelial growth factor (VEGF), Fn14 (which can react with Fn14 of human and mouse origins) were from Santa Cruz Biotech. Inc. (CA, USA); antibodies for NOX2 and Tweak were from Abcam (Cambridge, UK). Alexa-Flour goat anti-rabbit IgG and anti-mouse IgG were from Invitrogen (NY, USA).

Preparation of antisera. Anti-ALBH3 antisera were prepared as described previously against a synthetic PCA-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 enzyme-linked immunosorbent assay.

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 nM control RNA (Santa Cruz), siRNAs of human ALKBH3, Tweak or Fn14 (horseradish peroxidase [HP]-validated siRNA S102663892 for ALKBH3; S100056126 for Tweak; S103047621 for Fn14) from Qiagen or NOX2 from Santa Cruz using lipofectamine (Invitrogen, Tokyo, Japan) 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.

Preparation of cell lysates and Western blotting 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 h. We incubated the membranes with the indicated primary antibody for 1 h, and then incubated with horseradish peroxidase-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$_2$O$_2$ with dihydroethidium (DHE, Wako, Osaka, Japan) and 5,6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-DCF-DA, Wako), respectively. Cells were incubated with a 5 µM DHE or DCF-DA solution for 30 min at 37 °C. Excess DHE or DCF-DA was washed, then superoxide anion (O$_2^-$) or H$_2$O$_2$ formation in the cells was visualized using a Leica CTR6000 photomicroscope and quantitatively measured by flowcytometry.

**Cell cycle analysis.** We performed 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-d-old chick embryos. After a 24-h cultivation, we added control RNA or 10 µM siRNA for the indicated gene combined with atelocollagen (Atelogene, Koken Co., Ltd.) according to the manufacturer's protocol. Five days post-implantation, CAM samples were collected and fixed, then stained with anti-human cytokeratin antibody (CKAE1/AE3, DAKO, Tokyo, Japan) to allow invading cytokeratin-positive cells to be quantified. The depth of invasion from the CAM surface was defined as the leading front of three or more invading cells in five 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 three 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, Tokyo, Japan). After a 3-hr incubation period, optical absorbance at 490 nm was measured using a microplate reader. All experiments were performed 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-wk-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 $\mu$L medium were instilled into the bladder lumen and the urethra was ligated for 2 to 3 h. Fourteen days after the cell instillation, we transurethrally injected either control RNA ($n = 5$) or 10 $\mu$M of the ALKBH3 or Tweak siRNA + atelocollagen mixture ($n = 5$) previously described (Koken Co., Ltd.) into the urinary bladder (retain the RNA for 1 h).

**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 H&E and immunohistochemistry.

**Tissue samples and immunohistochemistry.** 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. Clinicopathologic data of the present cases were reviewed by two urological pathologists (K.S. and N.K., Department of Pathology, Nara Medical University Hospital) and summarized in supplementary Table 1. 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 (analysis of variance) or non-parametric tests (Mann-Whitney, Kruskal-Wallis) tests. All experiments were analyzed using a one-way analysis of variance and the Tukey post hoc test. A two-tail Student's t test was used to compare two data points. Graphical results represent mean ± S.E. Results were deemed statistically significant at the level of p < 0.05, and experiments were done with n ≥ 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 small interfering RNA (siRNA) for up to 72 hours (Fig. 1A). As shown in Figs. 1B and 1C, 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. Figs. 2A and 2B 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 analysis. Western blot demonstrated 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 chorioallantoic membrane (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 demonstrated 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 upper panel, ALKBH3 downregulation by *in vivo* transfection of siRNA, combined with atelocollagen, resulted in an ~6-fold decrease in tumor area. Expression of ALKBH3 downstream molecules, NOX2, Tweak, Fn14, and VEGF, was significantly reduced by ALKBH3 knockdown (Fig. 4A, lower 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 immunohistochemistry 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).

**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 figure 1A). 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 figure 1B). 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 performed 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...
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CIS, with both minimal and wide invasion (pT1) than in low-grade lesions with a noninvasive phenotype (pT0; supplementary figure2). Immunohistochemical analyses of Tweak and Fn14 showed the same results (Supplementary figure3). 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 histological grade or stage (supplementary figure4). Percentages of positive cells for ALKBH3 at initial diagnosis of pT0/pT1 cancers (n = 47) was statistically associated with multiplicity, tumor size (more or less than 3 cm), and the time of recurrence within 5 years, suggesting that immunoreactivity for ALKBH3 may be a useful marker for predicting malignant potential of urothelial carcinomas (supplementary figure5).

**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. Fig. 5A demonstrates 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 (pT1, ≥pT2) as well as high-grade CIS than in normal urothelium or noninvasive/low-grade urothelial carcinomas (pT0; Fig. 5B).

**Discussion**

We demonstrate 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 demonstrated 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 et al. reported that inhibition of ROS generation by NOX inhibitors suppressed invasion capacity by decreasing MMP-9 in prostate cancer cells. However, in 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 histological 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-1α in ovarian cancer cells (24). Abid et al. (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 \textit{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 \textit{in vivo}. Interestingly, Donohue et al. (26) recently reported that VEGF could directly induce Fn14 expression of endothelial cells. Although their experiments were not specific to 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 angiogenic factor; however, the correlation between Tweak/Fn14 and VEGF is not fully understood. Our present data are the first to demonstrate 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 \textit{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 two additional lines of evidence from previous reports, and the present study: (1) it has been shown that microvessel density is markedly increased in invasive urothelial carcinomas (27, 28); and (2) we showed that ALKBH3 expression in cancer cells and Tweak/Fn14 expression on both cancer and microvascular endothelial cells within cancer foci were dramatically increased in noninvasive urothelial carcinomas compared with invasive urothelial carcinomas, whereas NOX2 expression was not statistically correlated to pathological stage. Unfortunately, we have no data to explain the function of NOX2 in invasive urothelial carcinoma cells or the biological 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. Lastly, 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 figure 6). Thus, ALKBH3 can initiate effective angiogenesis through the manipulation of Tweak/Fn14 and VEGF, resulting in a highly advanced phenotype of urothelial carcinoma cells.
Acknowledgement:

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The Disclosure Statement, No conflict of interest
Figure legends:

Fig.1 Down-regulation 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. Expressions of ALKBH3 protein was examined by Western blotting (densitometric value (DV) 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 performed. Columns, mean; bars, SE ( *, statistically different from the value of control at p<0.01). Actin and GAPDH were used as internal controls throughout whole experiments.

Fig.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-DCFDA and DHE, and ROS generation was assessed by fluorescence microscopy (upper panel). UMUC2 and UMUC3 cells were transfected by ALKBH3 siRNA at 100 nM and control RNA. After 72 h inoculation, cells were treated with CM-DCFDA and DHE, then intracellular ROS levels were analyzed by flow cytometer (lower panel). B, Generation of hydrogen peroxide and oxygen radicals was quantitatively analysed by flow cytometer (left panel). And western blotting using anti-ALKBH3 or NOX2 antibody was performed (right panel)(DV 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 nM and control RNA. After 72 h inoculation, flow cytometric analysis using propidium iodide or western blotting using anti-NOX2 antibody was performed(DV control:siRNA: NOX2, 1:0.41 and 1:0.33 in UMUC2 and UMUC3 cells, respectively). Columns, mean; bars, SE ( * and #, statistically
different from the value of control at p<0.01 and p<0.001, respectively)

Fig.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-d-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 immunohistochemistry by cytokeratin AE1/AE3 are shown in A. Invasion depth and angiogenesis were analyzed (B, left panel), and cancer samples were resected and lysed, and expression of ALKBH3, Tweak, Fn14 or VEGF was examined in western blotting (B, right panel)(DV 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 tarsmected by Tweak, Fn14 or NOX2 siRNA at 100 nM and control RNA *in vitro*. After 72 h inoculation, expression of Tweak, Fn14, VEGF or NOX2 was analysed by western blotting(DV control:Tweak siRNA: Tweak, 1:0.41; Fn14, 1:0.25; VEGF, 1:0.18; NOX2, 1:1.18)(DV control:Fn14 siRNA: Fn14, 1:0.11; Tweak, 1:1.08; VEGF, 1:0.10; NOX2, 1:1.14) DV 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. *Columns*, mean; *bars*, SE (※ and #, statistically different from the value of control at p<0.01 and p<0.001, respectively)

Fig.4 Intravesical injection of ALKBH3 siRNA inhibits tumor growth *in vivo* in the mouse orthotopic bladder cancer implant model. A, fourteen days after KU7/GFP cells were transplanted into the mouse bladder, atelocollagen with either ALKBH3 siRNA or control RNA was transurethrally instilled into the bladder lumen. Bladders were resected 14 d post-instillation, and *in
situ images captured under fluorescent light and tumor area was measured by GFP expression (upper panel). Bladder cancer samples were resected and lysed, and expression of ALKBH3, NOX2, Tweak, Fn14 or VEGF was examined in western blotting (lower panel) (DV 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 immunohistochemistry by Fn14 or CD31 are shown in upper panel. 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. Columns, mean; bars, SE (#, statistically different from the value of control at p<0.001)

Fig.5 Tweak/Fn14 overexpression on endothelial cells is associated with invasion of urothelial carcinomas. A, Immunofluorescence by Tweak/Fn14 or CD31 was performed. 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.
References


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Fig. 1

(A) UMUC2  UMUC3
C  si  C  si
ALKBH3  actin
C, control RNA
si, ALKBH3 siRNA

(B) control RNA
ALKBH3 siRNA

UMUC2

UMUC3

Percentage of G1, S, or G2/M phase

UMUC2
control RNA
ALKBH3 siRNA

UMUC3
control RNA
ALKBH3 siRNA

Percentage of G1, S or G2/M phase

G1  S  G2/M
**Fig. 2**

(A) UMUC2

- Control
- ALKBH3 siRNA

(B) (%)

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</table>

- NOX2 siRNA

(-) (-) (+) (+)
Fig. 3

(A) KU7-GFP

Control ALKBH3 siRNA

Fluorescence
H&E
CK8/18

(B) Invasion depth (μm)

Invasion depth (μm)
Vascular branch

C, control RNA  si, ALKBH3 siRNA

(C) KU7

C

Tweak
Fn14
VEGF
NOX2
actin

C

Tweak
Fn14
VEGF
NOX2
actin

C

NOX2
VEGF
Fn14
actin

(D) Invasion depth (μm)

Invasion depth (μm)
Vascular branch

C

ALKBH3
Tweak
Fn14
VEGF
actin

C

NOX2
VEGF
Fn14
actin
Fig. 5

(A) DAPI CD31 Fn14/Tweak Overlay

B

1 / 2

p<0.001

(B) p<0.001

p<0.001

% vessels

Normal urothelium pTa pT1 >pT2 CIS

Normal urothelium pTa pT1 >pT2 CIS

% vessels

Tweak CD31/CD31 vessels

Tweak CD31/CD31 vessels

T

p<0.001

T

0 20 40 60 80 %

p<0.001

0 20 40 60 80 %

p<0.001

0 20 40 60 80 %

p<0.001

0 20 40 60 80 %
<table>
<thead>
<tr>
<th>Characterization of urothelial carcinomas</th>
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<tbody>
<tr>
<td><strong>Mean Age (years)</strong></td>
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<tr>
<td><strong>Gender</strong></td>
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<td><strong>Pathological stage</strong></td>
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<tr>
<td>pTis</td>
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<td>pTa</td>
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<td><strong>Grade</strong></td>
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<tr>
<td>Low grade</td>
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<td>High grade</td>
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ALKBH3 contributes to survival and angiogenesis of human urothelial carcinoma cells through NADPH oxidase and Tweak/Fn14/VEGF signals

Keiji Shimada, Tomomi Fujii, Satoshi Anai, et al.

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