Biochemical and Biophysical Research Communications 440 (2013) 731–736

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Inhibitory effect of Nodal on the expression of aldehyde dehydrogenase 1 in endometrioid adenocarcinoma of uterus

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ARTICLE INFO

Article history:
Received 20 September 2013
Available online 8 October 2013

Keywords:
Aldehyde dehydrogenase 1
TGF-β family
Nodal
Endometrioid adenocarcinoma

ABSTRACT

Cancers consist of heterogeneous populations. Recently, it has been demonstrated that cells with tumorigenic potential are limited to a small population, called cancer-initiating cells (CICs). Aldehyde dehydrogenase 1 (ALDH1) is one of the markers of CICs. We previously reported that ALDH1-high cases of uterine endometrioid adenocarcinoma showed poor prognosis, and ALDH1-high population of endometrioid adenocarcinoma cell line was more tumorigenic, resistant to anti-cancer drugs, and invasive than ALDH1-low population. Here, the regulatory signaling for ALDH1 was examined. The inhibition of TGF-β signaling increased ALDH1-high population. Among TGF-β family members, Nodal expression and ALDH1 expression levels were mutually exclusive. Immunohistochemical analysis on clinical samples revealed Nodal-high tumor cells to be ALDH-low and vise versa, suggesting that Nodal may inhibit ALDH1 expression via stimulating TGF-β signaling in uterine endometrioid adenocarcinoma. In fact, the addition of Nodal to endometrioid adenocarcinoma cell line reduced ALDH1-high population. Although ALDH1 mRNA level was not affected, the amount of ALDH1 protein appeared to be reduce by Nodal through ubiquitine–proteasome pathway. The regulation of TGF-β signaling might be a novel therapeutic target of CICs in endometrioid adenocarcinoma.

1. Introduction

Cancers consist of heterogeneous cell populations derived from a single clone. Recently, it has been demonstrated that cells with tumorigenic potential are limited to a small population, called cancer-initiating cells (CICs) [1,2]. The regulation of CICs by signaling pathways may be necessary for the effective therapeutic approaches against cancers. Several markers have been reported in CICs [3–11]. Among these, aldehyde dehydrogenase 1 (ALDH1) is supposed to be a general marker of various malignancies, such as leukemia, breast, brain, colon and lung cancers [7,11–16]. ALDH1 is a cytosolic enzyme responsible for oxidizing a range of aldehydes to their corresponding carboxylic acids, and involves in the degradation of toxins and the self-protection of cells [17].

Our recent report demonstrated that ALDH1 could be a marker of CICs in uterine endometrioid adenocarcinoma [18]. We detected that ALDH1 was expressed in a small portion of tumor cells, and these ALDH1-expressing cells were less mature than ALDH1-non-expressing cells. Moreover, in 98 cases of patients with endometrioid adenocarcinoma, higher ALDH1 expression showed poorer prognosis than those with lower expression. Multivariate analysis revealed high ALDH1 expression to be an independent poor prognostic factor. In endometrioid adenocarcinoma cell line, ALDH1-high cells were more invasive than ALDH1-low cells, and were highly resistant to anti-cancer drugs. These results suggest that ALDH1 is one of the CIC markers of uterine endometrioid adenocarcinoma, as in the case of other malignancies.

Few studies have been done on the signaling pathways regulating ALDH1 expression. Recently, Katsuno et al. [19] reported that ALDH1 expression is inhibited by TGF-β in diffuse-type gastric carcinoma. The regulatory effect of TGF-β signaling on CIC function is controversial. In breast cancer, glioma, and leukemia, TGF-β signaling promotes cancer progression through expansion of CICs [20–22]. On the other hand, TGF-β signaling reduces side-population fraction including CICs of diffuse-type gastric cancer and decreases CICs to inhibit tumorigenisis [19]. The effect of TGF-β signaling on CICs appears to depend on the tumor types. Here, the effect of TGF-β signaling on ALDH1 expression was examined in uterine endometrioid adenocarcinoma. We found that Nodal, a member of TGF-β family, inhibited ALDH1 expression via stimulating TGF-β signaling in uterine endometrioid adenocarcinoma.

Abbreviations: ALDH1, aldehyde dehydrogenase 1; CICs, cancer-initiating cells; DEAB, diethylaminobenzaldehyde.

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http://dx.doi.org/10.1016/j.bbrc.2013.09.139

2. Materials and methods

2.1. Cell line and Aldefluor assay

Endometrioid adenocarcinoma HEC-1 cell line was obtained from the Health Science Research Resources Bank of Osaka, Japan. Cells were cultured in DMEM (Wako, Osaka, Japan) supplemented with 10% FBS (Nippon Bio-Supply Center, Tokyo, Japan). The Aldefluor kit (StemCell Technologies, Vancouver, BC, Canada) was used to evaluate ALDH1-high population, according to the manufacturer's instruction. Briefly, cells were suspended in Aldefluor assay buffer containing the ALDH substrate BODIPY-aminoacetaldehyde and incubated for 45 min at 37 °C. The brightly fluorescent ALDH1-expressing cells were detected with FACS Canto II or FACS Aria II (BD Biosciences, Franklin Lakes, NJ, USA). As a negative control, cells were treated with 50 mM diethylaminobenzaldehyde (DEAB). FACS Canto II was used to detect the ALDH1-high population in HEC-1, and FACS Aria II was used for fluorescence-activated cell sorting. The evaluation of ALDH1-high population was done with or without LY36497 (Wako) and LDN193189 (Wako), recombinant human Nodal (rhNodal, R&D Systems, Minneapolis, MN, USA), and MG132 (Wako).

2.2. Immunohistochemistry

Histologic specimens for resected endometrioid adenocarcinoma were fixed in 10% formalin and routinely processed for paraffin-embedding. Paraffin-embedded specimens were stored in the dark room in the Department of Pathology of Osaka University Hospital at room temperature, sectioned at 4 μm thickness at the time of staining, and stained with hematoxylin and eosin and immunoperoxidase procedure. Expression of ALDH1, p-Smad3, Inhibin α, Inhibin β and Nodal was examined with anti-ALDH1 (BD Biosciences, ×200), anti-p-Smad3 (Abcam Ltd., Cambridge, UK, ×100), anti-Inhibin α (Abcam, ×200), anti-Inhibin β (Abcam, ×200), and anti-Nodal (Abcam, ×200) antibodies, respectively. The antigen retrieval with Pascal pressurised heating chamber (DAKO A/S, Glostrup, Denmark) was done for the staining of p-Smad3, Inhibin α, Inhibin β and Nodal. The sections were incubated with primary antibody, and then treated with ChemMate EnVision kit (DAKO). DAB (DAKO) was used as a chromogen. As the negative control, staining was carried out in the absence of primary antibody. In some experiments, double staining of ALDH1 and p-Smad3 was done with EnVision G/2 doublestain system (DAKO) according to the manufacturer's protocol. Firstly, the staining of ALDH1 was colored with DAB, and subsequently the staining of p-Smad3 was done with Permanent Red. Since the red fluorescence is released from Permanent Red, the signal of p-Smad3 was detected with fluorescence microscope (Biozero, Keyence, Osaka, Japan). Stained sections were evaluated independently by two pathologists (J.I. and E.M.). The study was approved by the ethical review board of Graduate School of Medicine, Osaka University.

2.3. Immunoblotting

Cells were washed with ice-cold PBS, and lysed using the buffer containing 200 mM Hepes, 5 M NaCl, 0.5 M EDTA, 0.1 M DTT, 50% Glycerol and 10% protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Electrophoresis was performed with 10% sodium dodecyl sulfate–polyacrylamide gels (ATTO, Tokyo, Japan) and proteins were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). Anti-ALDH1 antibody (×1000), anti-Nodal (×1000), and anti-actin antibody (Sigma, ×1000) were used as the primary antibody. HRP-conjugated anti-mouse IgG (H+L chain) (MBL, Nagoya, Japan, ×1000) or HRP-conjugated anti-rabbit IgG (H+L chain) (MBL, ×1000) was used as the secondary antibody.

2.4. Quantitative RT-PCR

Total RNA was extracted from cells using the RNeasy RNA extraction kit (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol. The cDNA was synthesized using oligo (dT) primers and SuperScript III reverse transcriptase (Life Technologies, Carlsbad, CA, USA). The qRT-PCR was performed with StepOnePlus™ Real-Time PCR instrument (Applied Biosystems, Foster City, CA, USA) using Taqman probe/primer sets specific for human ALDH1A1. GAPDH was used as a reference for gene amplification (Applied Biosystems).

2.5. Ubiquitination assay

The pRK5-HA-Ubiquitin-WT and its backbone vector pRK5 were obtained from Addgene. These plasmids were transiently transfected to HEC-1 cells using Lipofectamine® 2000 Reagent (Life Technologies) following the manufacturers' protocol. Forty-eight hours after transfection, cells were treated with 1 μM MG132 for 4 h, and lysed in buffer (200 mM Hepes, 5 M NaCl, 0.5 M EDTA, 0.1 M DTT, 50% Glycerol and 10% protease inhibitor cocktail, 10 μM MG132). Proteins that bound to HA-tagged ubiquitine were purified from the cell lysate with column chromatography using HA tagged Protein PURIFICATION KIT (MBL) following the manufacturers' protocol. Briefly, cell lysate was loaded to the column containing anti-HA tag beads, and the retained HA-tagged proteins were eluted with excess amount of HA pepitide. The original cell lysate, the eluted proteins (named as 'retained') and the proteins that were not retained in the column (named as 'flow-through') were immunoblotted with anti-ALDH1 antibody.

2.6. Proteasome activity assay

To measure the chymotrypsin-like, trypsin-like and caspase-like proteolytic activities of proteasome, the Proteasome-Glo™ Chymotrypsin-like, Trypsin-like and Caspase-like Cell-Based Assays (Promega, Madison, WI, USA) was used according to manufacturers instructions. Briefly, cells with or without the treatment of rhNodal (500 ng/mL) for 1 h were plated in white 96 wells plate with a transparent bottom and maintained overnight. Substrates for the chymotrypsin-like, trypsin-like and caspase-like activities (Suc-LLVY-aminoluciferin, Z-LRR-aminoluciferin and Z-nLPnlD-aminoluciferin, respectively) were added, and the plate was shaked at 700 rpm for 2 min. After 15 min of incubation, luminescence was measured by SH-9000Lab (Hitachi High Technologies, Tokyo, Japan).

2.7. Statistical analysis

Statistical analysis for experimental studies was carried out using Student's *t*-tests. The values are shown as the mean ± standard error (SE) of at least three experiments. The *p*-value $\leqslant 0.05$ was considered as statistical significance.

3. Results

3.1. Effect of TGF-β signaling pathway on ALDH1-high population

To examine the effect of TGF-β signaling pathway on ALDH1 enzymatic activity, the inhibitors of pathway, LY364947 and LDN193189, were added to HEC-1 cells, and Aldefluor assay was performed. TGF-β signaling is generally classified into two

pathways, which are mediated via Smad2/3 and via Smad1/5/8, respectively [23,24]. LY364947 inhibits the former [25] and LDN193189 inhibits the latter [26]. When LY362947 was added, the proportion of ALDH1-high population increased dose-dependently. In contrast, the addition of LDN193189 did not affect ALDH1-high population (Fig. 1A and B). These results suggested that the signal via Smad2/3 but not via Smad1/5/8 inhibited the expression of ALDH1. To confirm this, the double staining of phosphorylated Smad3 (p-Smad3, activated type of Smad3) and ALDH1 was carried out in the clinical samples of uterine endometrioid adenocarcinoma. Tumor cells with p-Smad3 did not express ALDH1, whereas those with ALDH1 did not express p-Smad3 (Fig. 1C). Mutually exclusive pattern of p-Smad3 and ALDH1 expression in clinical samples was consistent with the result that the signal via Smad2/3 inhibited ALDH1 expression in HEC-1 endometrioid adenocarcinoma cell line.

3.2. Inverse correlation of ALDH1 and Nodal expression

The inhibitory effect of Smad2/3 signal on ALDH1 expression suggested two possibilities; (1) ALDH1-high tumor cells expressed inhibitory factors on Smad2/3 signal, such as Inhibin (2) ALDH1-low tumor cells expressed stimulatory factors on Smad2/3, such as Activin and Nodal. Then, the expression of these factors was immunohistochemically examined in clinical samples of uterine endometrioid adenocarcinoma. Inhibin is composed of two subunits, Inhibin α and Inhibin β, whereas Activin is a dimer of Inhibin β. When stained with either anti-Inhibin α or Inhibin β antibody, no signals were detected (data not shown). This indicated that tumor cells of endometrioid adenocarcinoma did not express Inhibin and Activin. In contrast, Nodal expression was inversely correlated with the expression of ALDH1, showing the weak Nodal staining in tumor cells with strong ALDH1 staining and vice versa (Fig. 2A).

ALDH1-high and ALDH1-low HEC-1 cells were sorted separately, and Nodal expression was examined by immunoblot. The expression level of Nodal was significantly higher in ALDH1-low cells than in ALDH1-high cells (Fig. 2B), which was consistent with the inversely correlation of ALDH1 and Nodal expression in clinical samples.

3.3. Inhibitory effect of Nodal on ALDH1 expression

To examine the effect of Nodal on ALDH1 expression, rhNodal was added to HEC-1 cells for 30 min, and Aldefluor assay was done. The proportion of ALDH1-high cells decreased when rhNodal was added in a dose dependent manner (Fig. 3A). The addition of rhNodal did not affect the expression level of ALDH1 mRNA

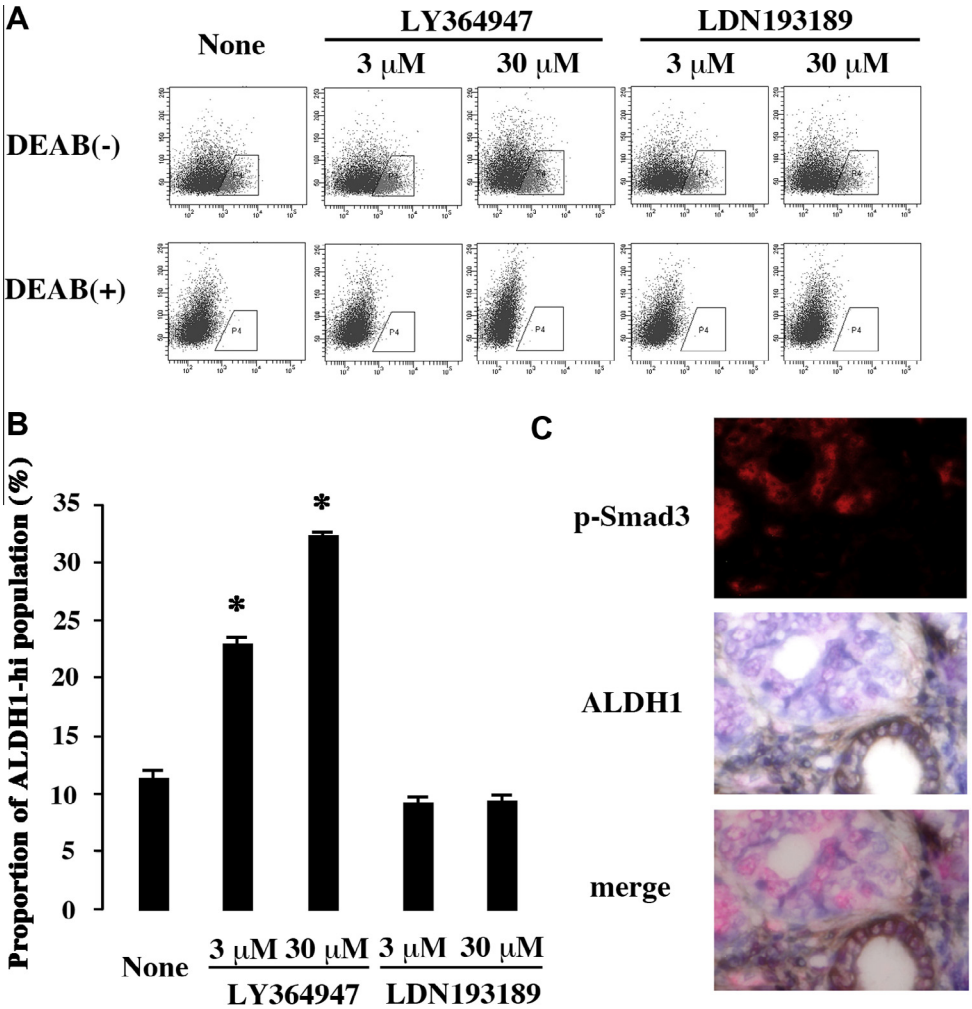

Fig. 1. Effect of TGF-β signal on ALDH1-high population. (A) Aldefluor assay. ALDH-high population was boxed. (B) Effect of TGF-β signal inhibitors on ALDH1-high population. (C) Double staining of p-Smad3 and ALDH1. Red fluorescence showed p-Smad3 expressing cells (upper), brown-stained cells ALDH1-high (middle). The merged image was shown (lower). Five cases of endometrioid adenocarcinoma were examined, and the representative result was demonstrated. $^{*}p < 0.05$ when compared to the value without inhibitors. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

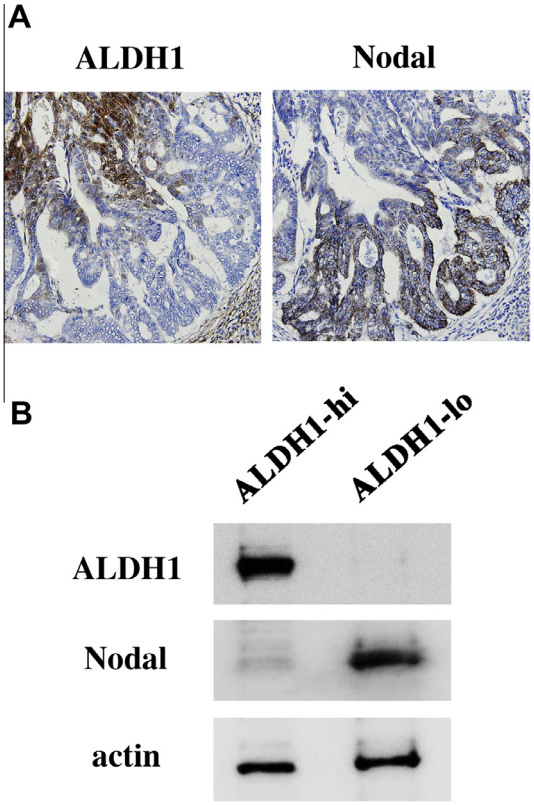

Fig. 2. Mutually exclusive expression pattern between ALDH1 and Nodal. (A) Immunohistochemistry of ALDH1 (left) and Nodal (right). ×400. Five cases of endometrioid adenocarcinoma were examined, and the representative result was demonstrated. (B) Immunoblot of the sorted ALDH1-high and ALDH1-low HEC-1 cells.

(Fig. 3B), suggesting that rhNodal did not stimulate ALDH1 mRNA transcription. To examine the possibility that rhNodal stimulated the degradation of ALDH1 protein, proteasome inhibitor MG132 was added. MG132 canceled the activity of rhNodal to reduce the proportion of ALDH1-high cells in a dose-dependent manner (Fig. 3C).

3.4. Effect of Nodal on proteasome activity

To examine the effect of Nodal on ALDH1 protein degradation, we first examined whether ALDH1 protein was ubiquitinated. The plasmid expressing HA-tagged ubiquitine was transfected to HEC-1 cells, and proteins with HA-tagged ubiquitine were purified using columns and blotted. Poly-ubiquitinated ALDH1 protein was detected in the column-retained fraction (Fig. 4A), indicating that ALDH1 was ubiquitinated. Next, the effect of Nodal on proteasome activities was examined. Caspase-like and chymotrypsin-like activities but not trypsin-like activity increased when Nodal was added (Fig. 4B). These results suggested that Nodal may increase the degradation of ALDH1 via ubiquitine–proteasome pathway.

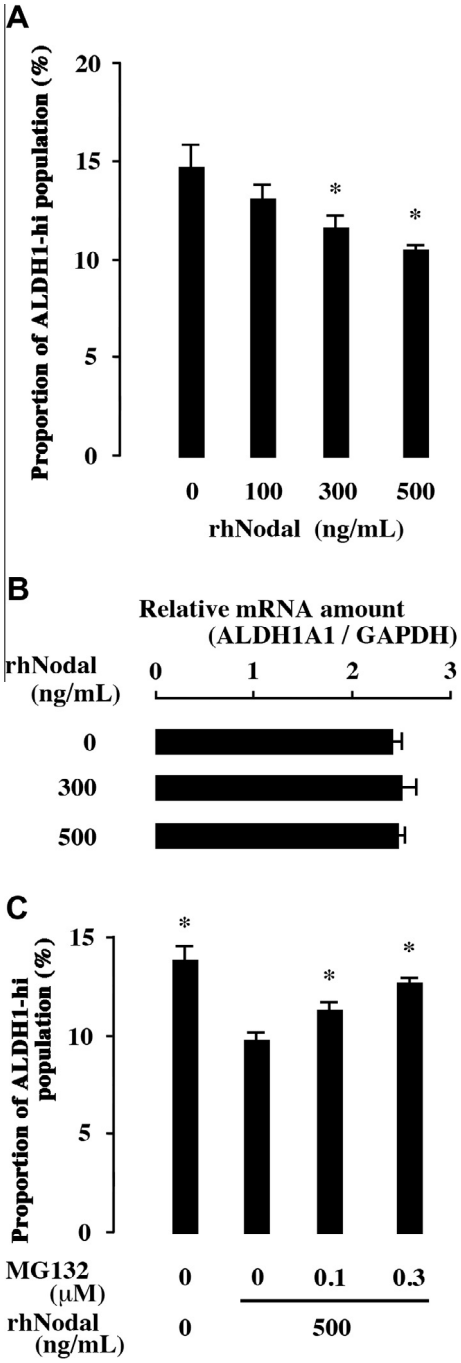

Fig. 3. Effect of rhNodal on ALDH1-high population. (A) Proportion of ALDH1-high population evaluated with Aldefluor assay. $^{*}p < 0.05$ when compared to the value without rhNodal. (B). Quantification of ALDH1A1 gene expression. (C) Effect of proteasome inhibitor MG132 on ALDH1-high population. $^{*}p < 0.05$ when compared to the value without MG132 in the presence of rhNodal.

4. Discussion

CICs are considered to be essential for tumor maintenance, recurrence and metastasis [1,2]. Therefore, eradication of CICs is essential to cure cancers. ALDH1 activity serves as a valuable functional marker for the identification of CICs, and increased ALDH1 activity is correlated to poor prognosis of several types of tumors [7,11–19]. We previously demonstrated that ALDH1 expression was correlated to T category, lymphatic invasion, recurrence and prognosis of patients in uterine endometrioid adenocarcinoma [18]. In this study, we examined the regulatory mechanism of ALDH expression, and found that Nodal, one of the TGF-β signal stimulators, reduced the proportion of ALDH1-high cells. Nodal plays the pro- or anti-tumorigenic roles in various types of cancers [27]. The acquisition of Nodal expression is associated with increased tumourigenesis, invasion and metastasis in malignancies such as glioma, melanoma, prostatic, and pancreatic carcinomas [28–35], whereas Nodal is capable of inducing apoptosis and inhibiting cell proliferation in ovarian cancer, breast cancer and

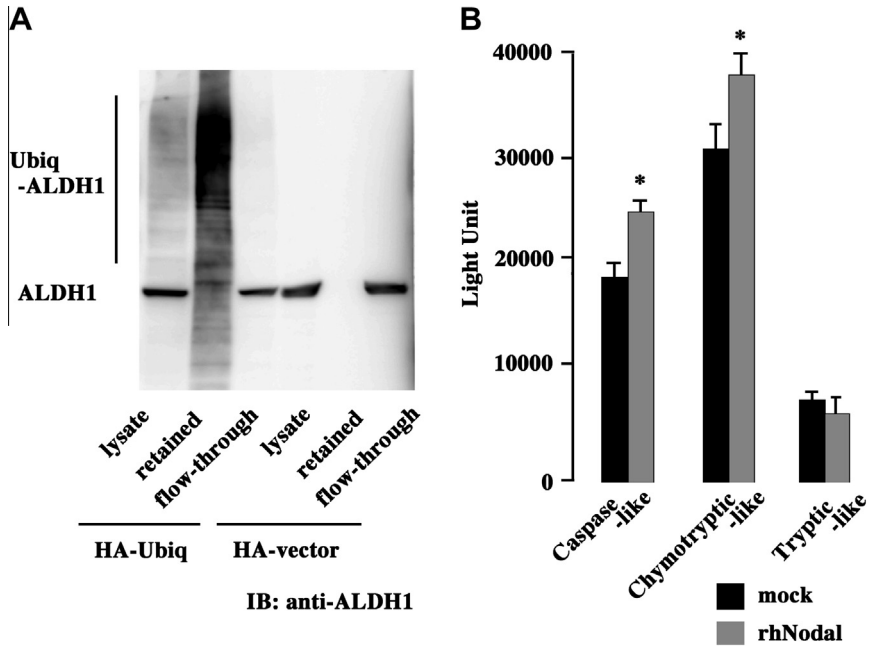

Fig. 4. ALDH1 ubiquitination and the effect of Nodal on proteasome activity. (A) HA-tagged ubiquitine was overexpressed in HEC-1, and the cell lysate was captured with column, in which HA-tagged proteins were retained. Cell lysate, retained portion, and the flow-through portion were blotted. Ubiquitinated ALDH1 was detected. (B) Effect of Nodal on proteasome activity. HEC-1 cells were treated with rhNodal, and their proteasome activities were examined. $^{*}p < 0.05$ when compared to the value without rhNodal.

choriocarcinoma [36–39]. The current result suggested that Nodal appeared to play the anti-tumorigenic role in endometrioid adenocarcinoma.

Based on our findings above, an important question that we have addressed is how Nodal inhibited ALDH1 expression. Nodal did not enhance ALDH1 mRNA transcription, which led us to the study on ALDH1 protein degradation. We showed that ALDH1 was a target for the ubiquitin–proteasome degradation pathway, and that Nodal increased the proteasome activity. Therefore, Nodal may increase the degradation of ALDH1 via activating the proteasome activity. Accumulating evidence reveals that the ubiquitin–proteasome system is involved in the regulation of fundamental processes in mammalian stem and progenitor cells of embryonic, neural, hematopoietic, and mesenchymal origin [40–42]. The population of cells identified by reduced proteasome activity is identical or overlapped with CICs and reduced 26S proteasome activity is a property of CICs that can cross species barriers [43]. These studies are consistent to our hypothesis that the inhibitory effect of Nodal on ALDH1 expression may due to the stimulatory effect of Nodal on proteasome.

On the relation of ALDH1 and TGF-β signaling, the similar finding to ours has been reported in diffuse-type gastric carcinoma-initiating cells; TGF-β signaling decreases ALDH1 expression and reduces CICs [19]. Therefore, these findings may offer a novel therapeutic approach for treating several cancers. However, the inhibitory effect of TGF-β on CICs is variable. In breast cancer, glioma, and leukemia, TGF-β signaling has been demonstrated to promote cancer progression through expansion of CICs [20–22]. Thus, further studies will be required to clarify the complex roles of TGF-β signaling on the regulation of CICs.

Acknowledgments

The authors thank Ms. Megumi Sugano, Ms. Etsuko Maeno and Ms. Takako Sawamura for their technical assistance. This work was supported by Grants from the Ministry of Education, Culture, Sports, Science and Technology, Japan (#23590425, #25460435, #25460436, #25108507), and from the Ministry of Healthy, Labor and Welfare, Japan (#KH24Q015b, #KH24Q059c).

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