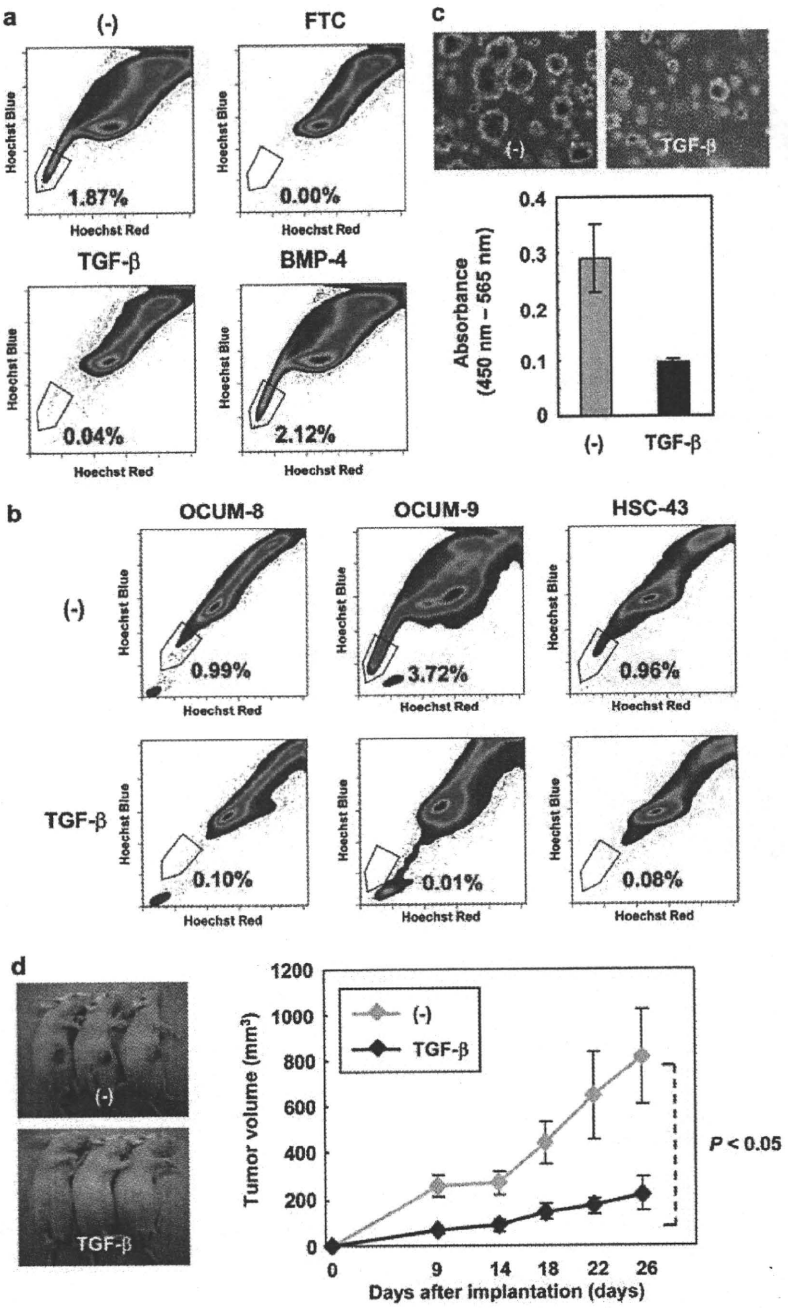

Figure 6 TGF-β decreases the SP fraction of OCUM-2MLN cells. (**a**) OCUM-2MLN cells were treated with TGF-β (1 ng/ml) or BMP-4 (30 ng/ml) for 72 h. Cells were stained with Hoechst 33342 and analyzed by flow cytometry. *Red numbers*, SP percentage of entire viable cell population. (**b**) Diffuse-type gastric carcinoma cells were treated with TGF-β (1 ng/ml) for 72 h. Cells were stained with Hoechst 33342 and analyzed by flow cytometry. *Red numbers*, SP percentage of entire viable cell population. (**c**) OCUM-2MLN cells were cultured with TGF-β (1 ng/ml) in soft agar, and the colony-forming ability was assessed. Representative photographs (*upper panels*) and numbers of colonies were (*lower panel*) indicated. *Columns*, mean of triplicate determinations; *bars*, s.d. (**d**) OCUM-2MLN cells were treated with TGF-β (1 ng/ml) for 72 h, and 1×10^4 cells were xenografted. Representative photographs (*left panels*) and tumor volumes (*right panel*) were indicated. *Points*, mean; *bars*, s.d.

not the other types of cells examined, exhibited tumorigenic ability (Figure 7d). These findings suggested that cancer cells originating from metastatic lesions expressed ABCG2 at high levels, correlated with the presence of SP cells and tumor-forming ability of cancer cells.

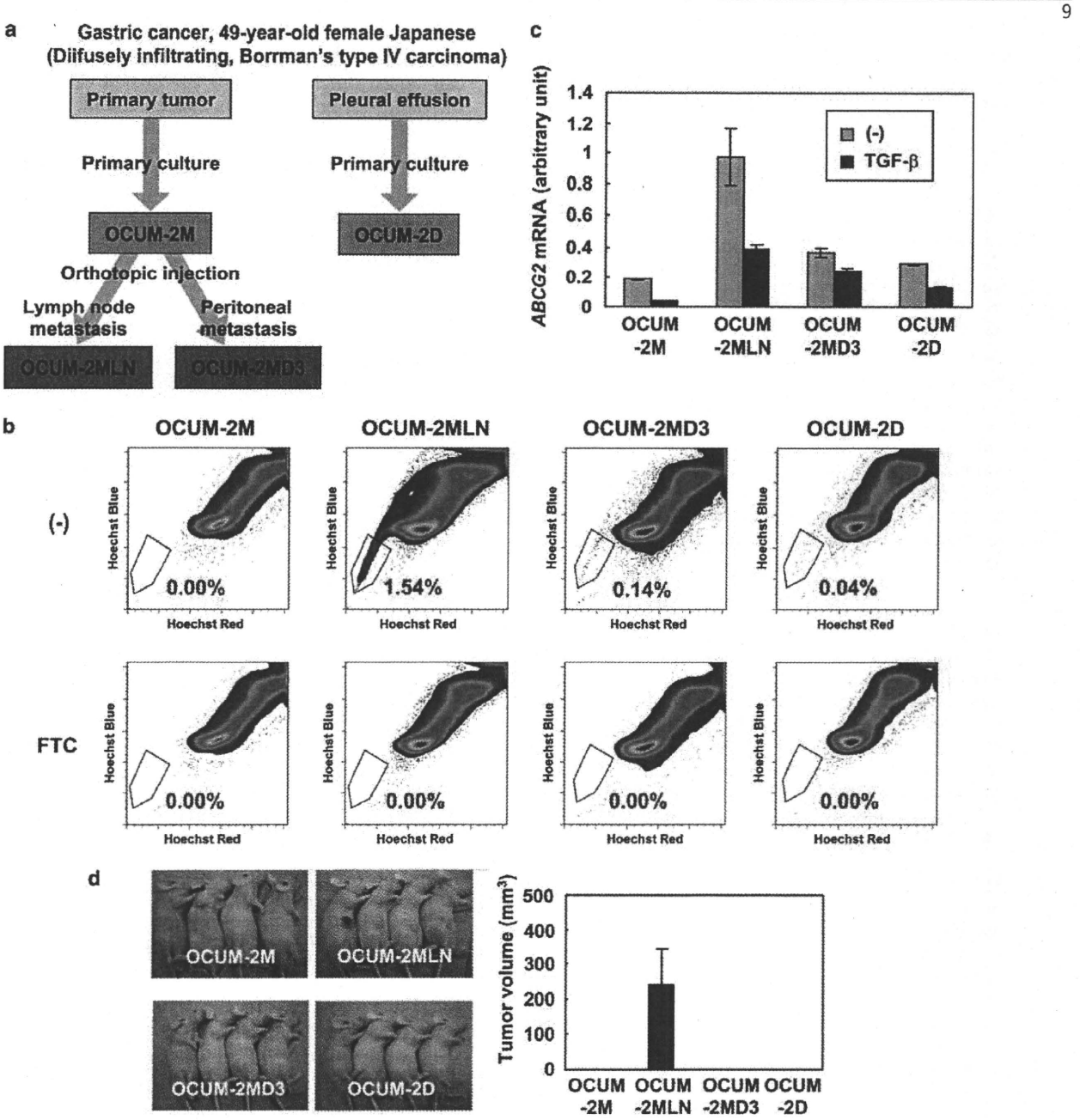

Figure 7 Highly metastatic cancer cells include more SP fractions. (**a**) Schematic representation of the *in vivo* process of selection of diffuse-type gastric carcinoma cells. (**b**) Diffuse-type gastric carcinoma cells were stained with Hoechst 33342 in the absence or presence of fumitremorgin C (FTC) and analyzed by flow cytometry. *Red numbers*, SP percentage of entire viable cell population. (**c**) Diffuse-type gastric carcinoma cells were treated with TGF-β (1 ng/ml) for 48 h. Expression of *ABCG2* mRNA was examined by quantitative real-time RT–PCR. *Columns*, mean; *bars*, s.d. (**d**) A total of 3×10^4 diffuse-type gastric carcinoma cells were xenografted. Representative photographs (*left panels*) and tumor volumes (*right panel*) were indicated. *Columns*, mean; *bars*, s.d.

Discussion

CICs are enriched in SP cells in diffuse-type gastric carcinoma

The existence of CICs within human gastric carcinoma was first detected by *in vitro* colony formation assay in 1981 (Laboisse *et al.*, 1981). Recently, a number of markers have proved useful for isolation of the subsets enriched in CICs in multiple cancers (Visvader and Lindeman, 2008). Some gastric cancer cells include subpopulations of $CD44^+$ cells, which exhibited tumorigenic ability (Takaishi *et al.*, 2009). However, no specific

marker for the diffuse-type gastric carcinoma-initiating cells has yet been established. In this study, we provide evidence that CICs are present in diffuse-type gastric carcinoma cells, and that the SP cells in OCUM-2MLN cells possessed repopulating capacity and high tumor-forming ability *in vivo*.

SP cells were initially identified in mouse bone marrow as long-term repopulating cells (Goodell *et al.*, 1997). This original discovery was followed by other reports on a wide variety of tissues (Zhou *et al.*, 2001). High expression of ABCG2 in hematopoietic stem cells was also reported (Scharenberg *et al.*, 2002). Moreover, recent studies have revealed that not only stem cells in normal tissues but also CICs are enriched in SP cells in many types of cancers (Wu and Alman, 2008). Our findings suggest that CICs in diffuse-type gastric carcinoma are enriched in SP fraction, and that expression of ABCG2 might serve as a marker for diffuse-type gastric cancer-initiating cells. As SP cells are estimated to be present at proportions between 0 and 20% of total cell population in many types of cancers (Dean *et al.*, 2005; Wu and Alman, 2008), it is reasonable that diffuse-type gastric cancer cells include SP cells in the range of 0–4% to total cancer cells.

Population of CICs is decreased by TGF-β: a novel mechanism of tumor suppression

TGF-β acts as tumor suppressors in many types of cancer. In gastrointestinal tumors, genetic and epigenetic inactivation of *TGFBR2*, *TGFBR1*, *SMAD4*/*MADH4* and *SMAD2*/*MADH2* has been found (Hahn *et al.*, 1996; Markowitz and Roberts, 1996; Grady and Markowitz, 2008). Consistent with these findings, we have shown that overexpression of dnTβRII in OCUM-2MLN cells caused the accelerated tumor formation *in vivo* in a mouse xenograft model (Figure 1d). However, under *in vitro* condition, phosphorylation of Smad2 in those cells was not detected in the absence of exogenous TGF-β, and detected only after the treatment of exogenous TGF-β (Figure 1a). Thus, TGF-β may be supplied from tumor microenvironment, for example, cancer-associated fibroblasts (Mizoi *et al.*, 1993), and have an influence on tumorigenicity of cancer cells.

Interestingly, TGF-β altered the percentage of CICs within diffuse-type gastric carcinomas. As TGF-β suppressed the expression of ABCG2 in other types of cancer cells (Figure 4), it is possible that the percentages of CICs in other types of cancers are also decreased by TGF-β. However, contrary to the present findings for diffuse-type gastric carcinomas, TGF-β was reported to maintain the 'stemness' of glioblastoma-initiating cells (Ikushima *et al.*, 2009; Peñuelas *et al.*, 2009). TGF-β also maintains the stem-cell-like properties of leukemia-initiating cells in chronic myeloid leukemia through regulation of AKT activation and FOXO3a localization (Naka *et al.*, 2010). TGF-β might thus have different, tissue-dependent regulatory effects on CICs.

We also examined the effect of BMP-4, another member of TGF-β family, on the expression of ABCG2 and the maintenance of SP cells within diffuse-type gastric carcinoma. In several types of cancers, BMP ligands are estimated as a novel therapeutic agent, which can induce 'differentiation' of cancer stem cells, attenuate the tumor-forming ability of cancer, and may be used to prevent growth and recurrence of cancers (Piccirillo *et al.*, 2006; Sneddon *et al.*, 2006; Lee *et al.*, 2008). However, BMP-4 reduced neither the expression of ABCG2 in OCUM-2MLN cells nor population of SP cells in these cells (Figure 3a, b and 6a).

TGF-β regulates the expression of ABCG2 and drug efflux ability

ABCG2, also termed BCRP, is a 72 kDa half-transporter containing six putative transmembrane α-helices (Velamakanni *et al.*, 2007; Gradhand and Kim, 2008), and is a member of subfamily G of the ABC transporters, expressed in various types of cancers. ABCG2 is known to be responsible for the efflux of chemotherapeutic drugs. Thus, pharmacological inhibition of ABCG2 activity with selective inhibitors was tested in an attempt to overcome ABCG2-mediated drug resistance. In addition, siRNA targeting ABCG2 expression in cancer cells was also designed. However, the mechanism of regulation of ABCG2 expression in cancer cells have yet to be fully understood. Here, we found that ABCG2 expression was repressed by TGF-β in several types of cancers, including diffuse-type gastric carcinoma. These findings are in agreement with the observations using human breast cancer MCF7 cells (Yin *et al.*, 2008). TGF-β appears to directly regulate the expression of ABCG2 in diffuse-type gastric carcinoma cells. Moreover, we have demonstrated that Smad complex directly binds to *ABCG2* promoter/enhancer in OCUM-2MLN cells and that TGF-β negatively regulates the transcription of *ABCG2* in these cells (Figure 3e). This is consistent with our recent chromatin immunoprecipitation-chip analysis data, which indicated that Smad2/3 complex directly binds to the transcription start point of the *ABCG2* locus in the genome of human normal keratinocyte, HaCaT cells (Koinuma *et al.*, 2009 and our unpublished data).

Metastasis of diffuse-type gastric carcinoma might depend on a distinct population of tumor cells

Recent findings have suggested that in some cancers metastasis arises directly from CICs. Pancreatic cancer stem cells, profiled as $CD133^+$ $CXCR4^+$, exhibited significantly stronger migratory activity *in vitro* (Hermann *et al.*, 2007). Furthermore, $CD44^+$ $CD24^{-/low}$ cells, a cancer-initiating subset of breast cancer, are readily detectable in pleural effusions in breast cancer patients (Al-Hajj *et al.*, 2003). Moreover, peritoneal metastasis of diffuse-type gastric carcinoma depends on the adhesive ability of cancer cells, and the adhesive ability of SP cells was reported to be significantly higher than that of parental cells (Nishii *et al.*, 2009). This study demonstrated that OCUM-2MLN and OCUM-2MD3 cells, both of which were isolated from metastasis arising in OCUM-2M cell-xenografted mice, expressed higher levels of ABCG2 and included more SP cells than

parental OCUM-2M cells. When equal numbers of OCUM-2M or OCUM-2MLN cells were orthotopically injected, lymph node metastases were only observed in the OCUM-2MLN-injected mice (Fujihara *et al.*, 1998). Intraperitoneal inoculation of OCUM-2MD3 cells caused peritoneal metastases in all treated mice, whereas that of OCUM-2M cells failed to induce peritoneal metastasis (Yashiro *et al.*, 1996). Taken together, these findings suggest that OCUM-2MLN and OCUM-2MD3 cells, which may include relatively large SP populations, have stronger metastatic ability than OCUM-2M cells. Metastasis of diffuse-type gastric carcinoma might be derived from a minority of tumor cells, and complete eradication of this minor population may be necessary for the effective treatment of cancer. Alternatively, induction of differentiation of the CICs by activation of TGF-β signaling pathway may be another possibility for eradication of this minor population.

Diffuse-type gastric carcinoma is characterized by its thick stromal fibrosis, thus, also known as linitis plastica. Although TGF-β produced by cancer cells and/or by cancer-associated fibroblasts enhances the fibrosis, the role of TGF-β in the development of diffuse-type gastric carcinoma still remains controversial (Mizoi *et al.*, 1993; Mishra *et al.*, 2005). Either increased or decreased survival in diffuse-type carcinoma patients were reported to correlate with the expression of TGF-β (Kinugasa *et al.*, 1998; Vagenas *et al.*, 2007). However, our present findings suggest that TGF-β suppresses the progression of tumor by induction of the differentiation of CICs of this type of cancer. Study of the interaction between CICs and the tumor microenvironment mediated through TGF-β signaling should provide additional insights into the management of cancer.

Materials and methods

Cell culture

Human diffuse-type gastric carcinoma cells were cultured as shown in Supplementary Table S1 (Takemura *et al.*, 2004; Yashiro *et al.*, 2009; Yanagihara *et al.*, 1993). Lentivirus vectors were used to generate 2MLN-dnTbRII and control 2MLN-GFP cells as described (Komuro *et al.*, 2009). A human keratinocyte cells, HaCaT, and a human breast cancer cells, MDA-MB-231, were cultured as described (Ehata *et al.*, 2007a). Human non-small-cell lung carcinoma cells, A549, human hepatocellular carcinoma cells, HuH7, and human cervical carcinoma cells, HeLa, were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin/streptomycin.

siRNA

siRNA duplex oligoribonucleotides against human Smad4 (Stealth RNAi VHS41118) or control siRNA (Stealth RNAi 12935-200) were synthesized by Invitrogen (Carlsbad, CA, USA). OCUM-2MLN cells were transfected in the presence of 125 pmol of either siRNA/Smad4 or control siRNA in a 500 μl volume with 8 μl Lipofectamine 2000 (Invitrogen) per well of a 6-well plate according to the manufacturer's protocols. To confirm knockdown of Smad4, cells were harvested 24 h after siRNA transfection and subjected to quantitative real-time RT–PCR.

RT–PCR

Quantitative real-time RT–PCR was performed as described (Ehata *et al.*, 2007b). Values were normalized to hypoxanthine guanine phosphoribosyl transferase 1 (HPRT1). All samples were run in duplicate. The primer sequences were listed in Supplementary Table S2.

Immunoblotting

Immunoblotting was performed as described (Ehata *et al.*, 2007b; Komuro *et al.*, 2009). Anti-phospho-phosphorylated RB antibody was obtained from BD Pharmingen (San Jose, CA, USA). Anti-ABCG2/BCRP antibody was obtained from Millipore (Billerica, MA, USA). LAS-4000 Image Analyzer (Fuji Photo Film, Kanagawa, Japan) was used for the detection of immunoblotted proteins.

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed as described (Koinuma *et al.*, 2009). Whole-cell extracts were incubated at 4 °C for 8 h with Dynabeads sheep anti-mouse IgG (Invitrogen) that had been preincubated with 10 μg of anti-Smad2/3 antibody (Cell Signaling Technology, Beverly, MA, USA) in phosphate-buffered saline/0.5% bovine serum albumin. Genomic DNA was then extracted with a PCR Purification Kit (Qiagen, Valencia, CA, USA), eluted in 100 μl of Tris-EDTA, and used for quantitative real-time PCR. Hemoglobin beta (HBB) and plasminogen activator inhibitor type 1 (PAI-1) were used for negative and positive control, respectively. The primer sequences were listed in Supplementary Table S2.

Cell proliferation assay

Cell proliferation assay was performed as described (Ehata *et al.*, 2007a). Briefly, HaCaT and OCUM-2MLN cells were seeded in duplicate at a density of 2.5×10^4 cells per well in 24-well plates. On the following day, cells were treated with TGF-β (1 ng/ml) for 4 days. Cells were trypsinized and counted with hemocytometer.

Colony formation assay in soft agar

Agar (Nacalai Tesque, Kyoto, Japan) was dissolved in culture medium to 0.5% and plated in 6-well plates (bottom layer). Then, cells were seeded at $1–2 \times 10^4$ cells per well in 0.3% agar (top layer) over bottom layer. Cells were covered with liquid growth media containing TGF-β, and cultured for 3 weeks. Cell viability was measured using Cell Count Reagent SF (Nacalai Tesque).

Flow cytometric analysis and sorting

Flow cytometric analysis and sorting were performed as described (Katayama *et al.*, 2009). Briefly, cells were resuspended at a concentration of 1×10^6 cells per ml in ice-cold Hank's balanced salt solution supplemented with 2% fetal bovine serum and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. These cells were treated with 1–12 μg/ml Hoechst33342 (Invitrogen) for 60 min at 37 °C either alone or in the presence of inhibitors. After washing with phosphate-buffered saline, 3×10^4 cells were analyzed using a FACS Vantage SE flow cytometer (BD Biosciences, San Jose, CA, USA). Hoechst 33342 was excited with the UV laser at 350 nm and fluorescence emission was measured with 405/BP30 (Hoechst blue) and 570/BP20 (Hoechst red) optical filters. SP gate was defined as diminished region in the presence of reserpine or fumitremorgin C. Analysis was done using Flow Jo software (Treestar, Ashland, OR, USA).

Mouse xenograft model and in vivo *gene expression analysis*

Animal experiments using a mouse xenograft model were performed (Komuro *et al.*, 2009). For the xenografts of sorted

cells, Matrigel (BD Bioscience) was used. The significance of differences was determined by repeated-measures analysis of variance test, with *P*-values less than 0.05 considered significant. *In vivo* gene expression analysis was performed as described (Komuro *et al.*, 2009). Briefly, tissue samples from subcutaneous 2MLN-GFP or 2MLN-dnTβRII tumors were digested with collagenase and trypsinized. The resulting single-cell suspension was subjected to magnetic cell sorting with magnetic microbeads conjugated to CD326 antibody (Miltenyi Biotec, Sunnyvale, CA, USA) to separate CD326-positive human cancer cells from CD326-negative mouse stromal cells. Total RNAs were purified with the RNeasy Mini Kit (QIAGEN) and used for oligonucleotide microarray, GeneChip Human Genome U133 Plus2.0 (Affymetrix, Inc. (Santa Clara, CA, USA)). MultiExperiment Viewer Version 4.6 software (Institute for Genomic Research) was used for the statistical analysis.

Conflict of interest

The authors declare no conflict of interest.

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Context-dependent regulation of the expression of c-Ski protein by Arkadia in human cancer cells

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Arkadia is a positive regulator of transforming growth factor-β (TGF-β) signalling, which induces ubiquitylation and proteasome-dependent degradation of negative regulators of the TGF-β signalling pathway, *i.e.* Smad7, c-Ski and SnoN. In the present study, we examined the roles of Arkadia in human cancer cells. We first examined the expression of Arkadia in 20 cancer cell lines and 2 non-cancerous cell lines, and found that it was expressed ubiquitously at both the mRNA and protein levels. Interestingly, levels of expression of c-Ski protein, one of the substrates of Arkadia, were not correlated with those of c-Ski mRNA. Arkadia induced down-regulation of c-Ski protein expression in many cell lines examined, but did not in certain cell lines with high levels of expression of c-Ski protein. We also found that knockdown of Arkadia attenuated the induction of TGF-β target genes, whereas ectopically expressed Arkadia enhanced it. Notably, over-expression of Arkadia inhibited the growth of HepG2 cells in the presence as well as the absence of TGF-β stimulation. Arkadia thus regulates the levels of expression of c-Ski protein in cell-type-dependent fashion, and exhibits a tumour suppressor function by inhibiting tumour cell growth.

Keywords: cancer/degradation/Ski/TGF-β/ubiquitin ligase.

Abbreviations: CBP, CREB-binding protein; E3, ubiquitin-protein isopeptide ligase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, haemagglutinin; HDACs, histone deacetylases; MCS, multi-cloning site; MEF: mouse embryonic fibroblast; PCR, polymerase chain reaction; PAI-1, plasminogen activator inhibitor-1; RT, reverse transcription; Sno, Ski-related novel gene; TGF-β, transforming growth factor-β.

Transforming growth factor-β (TGF-β) has a diverse array of activities, including growth inhibition, regulation of motility, extracellular matrix production, differentiation and apoptosis, in various target cells (*1*). TGF-β signalling must be tightly controlled, since its abnormality has been reported to cause progression of various diseases, including cancer and fibrosis (*2*). TGF-β plays dual roles in the progression of cancer (*3*, *4*). In the early stages of carcinogenesis, TGF-β acts as a tumour suppressor by inhibiting cell growth. In contrast, TGF-β exerts tumour-promoting effects by inducing invasion and metastasis in advanced stages of cancer. Levels of expression of TGF-β are positively correlated with clinical stage in certain tumours.

TGF-β signal is transduced through two distinct serine–threonine kinase receptors, termed type I and type II (*5–8*). Upon binding of TGF-β to type II receptor, type I receptor is recruited to the ligand–receptor complex and is phosphorylated by the constitutively active type II receptor kinase. Type I receptor is then activated, and phosphorylates receptor-regulated Smads (R-Smads), Smad2 and Smad3. Phosphorylated Smad2 and Smad3 form complexes with Smad4, a common-mediator Smad (co-Smad), and translocate into the nucleus. The activated Smad complexes then bind to promoter regions of target genes either directly or together with other transcription factors, and regulate their transcription in collaboration with transcriptional co-activators and co-repressors (*7*, *9*).

Arkadia was originally identified by gene-trap mutagenesis in mice as a factor required for induction of the mammalian node in extraembryonic lineages (*10*), and was found to induce mesendoderm by enhancing nodal-related signalling (*11*). Arkadia is a nuclear protein with 989 amino acid residues, including a characteristic RING domain at its C-terminus. We previously found that Arkadia is an E3 ubiquitin ligase that enhances TGF-β signalling by targeting negative regulators, *i.e.* c-Ski/SnoN and Smad7 (*12*, *13*).

c-Ski and SnoN are members of the Ski family of oncoproteins (*9*, *14*). Ski was originally identified as the transforming protein (v-Ski) of the avian retrovirus that induces oncogenic transformation of chicken embryo cells (*15*). The Ski family of nuclear oncoproteins represses TGF-β signalling principally through interaction with Smad proteins (*16*, *17*). c-Ski and SnoN interact with Smad2/3 and Smad4 in activated Smad complexes (*18*, *19*). They also bind directly to mSin3A and N-CoR and form a complex containing histone deacetylases (HDACs), thus repressing transcription (*20*). In addition, c-Ski and SnoN have been shown to compete with transcriptional co-activator p300 and/or CREB-binding protein (CBP) for binding to Smad complexes (*18*, *19*, *21*) and to stabilize inactive Smad complex on the promoter regions of target genes

(*22*). In contrast, Smad7, an inhibitory Smad (I-Smad), competitively inhibits phosphorylation of Smad2 and Smad3 through binding to activated type I receptor kinase of TGF-β in the cytoplasm (*23*, *24*).

Misexpression of these negative regulators has been implicated in various pathological conditions. Increased expression of Smad7 has been found in inflammatory bowel disease (*25*) and pancreatic cancer (*26*). Reduction of Smad7 protein has been reported in human fibroblasts of patients with scleroderma (*27*) and in tissues with renal fibrosis in mice (*28*). Increased expression of SnoN or c-Ski has been implicated in the progression of oesophageal squamous cell carcinomas (*29*, *30*), melanomas (*31*), estrogen-receptor-positive breast carcinomas (*32*) and colorectal carcinomas (*33*). Some of these cancers exhibit gene amplification of c-Ski or SnoN (*29*, *33*, *34*). Since increased expression of c-Ski or SnoN has been reported to be associated with poor prognosis, overactivity of SnoN and c-Ski may cause cancer. In contrast, systemic deletion of one copy of the *Sno* or *Ski* gene causes increased susceptibility to chemical carcinogens (*35*, *36*). Control of the levels of expression of these negative regulators within appropriate ranges thus appears to be important.

Arkadia appears to play important roles in cancers through regulation of the protein expression of c-Ski/SnoN and TGF-β signalling. However, the roles played by Arkadia in tumours have yet to be fully determined. We describe here the relationship between expression of Arkadia and that of c-Ski/SnoN, as well as the roles played by Arkadia in tumour cells.

Materials and Methods

Cell culture

Cells were cultured in the medium shown in Supplementary Table I, in a 5% CO_2-humidified atmosphere at 37°C.

Lentiviral production and infection

Lentivirus expression vectors (*37*) for Arkadia and multi-cloning site (MCS) were constructed as previously described (*38*). Briefly, haemagglutinin (HA)-tagged mouse Arkadia or MCS was inserted into pENTR vectors (Invitrogen), and then transferred to pCSII-EF-RfA vectors using LR clonase (Invitrogen). 293FT cells (6×10^6 cells; Invitrogen) were transfected using Lipofectamine 2000 (Invitrogen) with pCSII-EF-RfA containing Arkadia or MCS, pCAG-HIVgp and pCMV-VSV-G-RSV-Rev. The culture supernatants were collected 72 h after transfection and used for transduction of HepG2 and OCUM-2MLN cells. HepG2 cells were infected with the lentivirus twice.

RNA interference

RNA interference using siRNA oligonucleotides was performed as described below. Sequences of RNA oligonucleotides used to knock down human Arkadia, human c-Ski and human SnoN were as follows: siArkadia RNF111-HSS123238 (forward, 5′-UAACACUUC UCGUUUCUUCCUCUGC-3′; reverse, 5′-GCAGAGGAAGAAA CGAGAAGUGUUA-3′), siArkadia RNF111-HSS123240 (forward, 5′-AACACAAUUCUGCACAUACGAAGGG-3′; reverse, 5′-CCCUUCGUAUGUGCAGAAUUGUGUU-3′), sic-Ski SKI-HSS109772 (forward, 5′-UUGUGCGAGUGCACCACGAACUU GU-3′; reverse, 5′-ACAAGUUCGUGGUGCACUCGCACAA-3′) and siSnoN SKIL-HSS109774 (forward, 5′-AAUAAACCCUGAC AUUUGCCUAGGC-3′; reverse, 5′-GCCUAGGCAAAUGUCAG GGUUUAUU-3′). For knockdown of Arkadia, RNF111-HSS123238 or RNF111-HSS123240 was used. Similar knock-down efficiency was confirmed with these two siRNAs. Pre-annealed oligonucleotides (Stealth RNAi oligonucleotides) were obtained from Invitrogen. Oligonucleotides for negative controls were also purchased from Invitrogen. Transfection of these oligonucleotides was performed using HiPerFect transfection reagent (Qiagen) at the same time as seeding of cells. Oligonucleotides were used at final concentrations of 50 nM or 100 nM for silencing of Arkadia expression, with 50 nM used for SnoN expression and 100 nM for c-Ski expression. Cells were cultured 40 or 60 h (for MKN45 cells) before analysis.

Immunoblotting

MDA-MB-231 cells and mouse embryonic fibroblasts (MEFs) (*39*) were treated with 1 ng/ml TGF-β (TGF-β1, R & D Systems) before analysis, where indicated. Cells were lysed with a buffer containing 1% Nonidet P-40, 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM phenylmethylsulphonyl fluoride, 1% Trasyrol, 50 μM MG132 (Peptide Institute) and 5 mM EDTA. Cleared cell lysates were separated by SDS–PAGE and transferred to Fluoro Trans W membrane (Pall). Immunoblotting was performed as described previously (*13*) using the following antibodies: anti-Arkadia 3AP4 (*13*) for simultaneous detection of both endogenous human Arkadia and exogenous mouse Arkadia, anti-RNF111 (Abnova) for immunoblotting of endogenous Arkadia in human cell lines, anti-c-Ski (Millipore) for detection of endogenous c-Ski in human cell lines and in MEFs, anti-SnoN H-317 (Santa Cruz Biotechnology) for immunoblotting of endogenous SnoN, and anti-tubulin DM 1A (SIGMA). Bands of immunoblotting were quantified using Quantity One 1-D Analysis software (Bio-Rad Laboratories).

Semi-quantitative RT–PCR

Total RNAs from wild-type and *Arkadia*$^{-/-}$ MEFs were extracted using the RNeasy Mini Kit (Qiagen). Reverse transcription and semi-quantitative RT–PCR was performed as described previously (*13*). The primer sequences used for detection of mouse c-Ski were: forward, 5′- GAGGGTGCCCCGGGTCTCAG-3′; reverse, 5′- AC GGTGGTGCAGGGTGGACT-3′.

Real-time RT–PCR

Total RNA from HEK293, HaCaT, and 20 tumour cell lines was prepared using the RNeasy Mini Kit. cDNA synthesis was performed as described previously (*13*). Quantitative RT–PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) or FastStart Universal SYBR Green Master [Rox] (Roche) and a 7500 Fast Real-Time PCR System (Applied Biosystems). The primer sequences used were as follows: human SnoN (forward, 5′-CTGTGTTGGAAGGGGAATCT-3′; reverse, 5′-TTTGCTGGAGTGTAAATTCTCG-3′) and human $p15^{INK4b}$ (forward, 5′-GCCGCCCACAACGACTTTAT-3′; reverse, 5′-GCT TGCAGGCTTACAGGCTTTC-3′). Primers for human Arkadia, human Smad7, and human GAPDH were previously described (*12*). Primers for human c-Ski, human plasminogen activator inhibitor-1 (PAI-1), and human $p21^{WAF}$ were also previously described (*40*).

Results

Accumulation of c-Ski protein by knockdown of Arkadia

We previously reported that ectopic expression of Arkadia induces ubiquitylation and proteasome-dependent degradation of c-Ski (*13*). To determine whether endogenous Arkadia affects expression of c-Ski protein, we compared the levels of expression of c-Ski protein in Arkadia-knocked-down and control MDA-MB-231 cells. Since c-Ski has been reported to undergo degradation in response to TGF-β stimulation (*41*), we also compared the time courses of protein expression of c-Ski after TGF-β stimulation. As shown in Fig. 1A, c-Ski protein accumulated in Arkadia-silenced cells in the absence of TGF-β stimulation, suggesting that Arkadia induces c-Ski degradation under resting conditions. Upon stimulation with TGF-β,

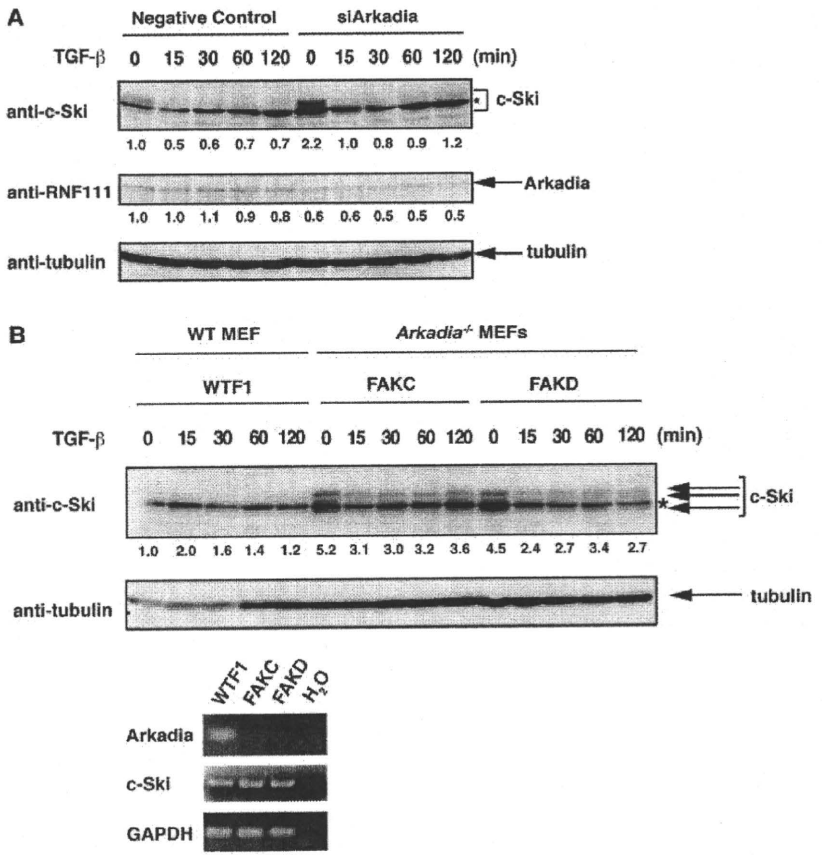

Fig. 1 Arkadia contributes to degradation of c-Ski protein. (**A**) Knockdown of Arkadia was performed by siRNA. MDA-MB-231 cells were transfected with siArkadia oligonucleotide or negative control oligonucleotide at the same time as seeding. Cells were treated with 1 ng/ml TGF-β and harvested at the indicated time points. Cell lysates were subjected to SDS–PAGE, followed by immunoblotting using anti-c-Ski antibody (top panel), anti-RNF111 (Arkadia) antibody (middle panel) and anti-tubulin antibody (bottom panel). Asterisk denotes non-specific bands. The specific bands of c-Ski and Arkadia were quantified and normalized to those of tubulin. Values shown at the bottom of corresponding panels were relative to those of negative control at 0 min. (**B**) Wild-type (WT) MEF cell line (WTF1) and *Arkadia*$^{-/-}$ MEF cell lines (FAKC and FAKD) were treated with 1 ng/ml TGF-β and harvested at the indicated time points. Time courses of expression of c-Ski protein upon TGF-β stimulation were examined (top panel). Asterisk denotes non-specific bands. The specific bands of c-Ski were quantified and normalized to those of tubulin. Values shown at the bottom of top panel were relative to that of WT MEF at 0 min. Expression of Arkadia and c-Ski mRNA are shown at the bottom.

c-Ski protein was degraded within 15 min in the control cells, consistent with previous reports (*41*). TGF-β-induced degradation was not abrogated in Arkadia-knocked-down cells, suggesting the possibility of involvement of E3 ubiquitin ligases other than Arkadia. We also performed a similar experiment using *Arkadia*$^{-/-}$ and wild-type MEFs, and obtained similar results (Fig. 1B). c-Ski protein was hardly detected in wild-type MEFs, but accumulated in *Arkadia*$^{-/-}$ MEFs, whereas expression levels of c-Ski mRNA were similar between WT and *Arkadia*$^{-/-}$ MEFs. c-Ski protein was significantly reduced 15 min after TGF-β stimulation in *Arkadia*$^{-/-}$ MEFs. These findings suggest that Arkadia is involved in the degradation of c-Ski protein in the absence of TGF-β stimulation. Although Arkadia may also play a role in TGF-β-induced degradation of c-Ski, other ubiquitin ligases may also play a role in it.

Broad expression of Arkadia in various cancer cell lines

TGF-β has two opposing effects on the progression of cancer. Since Arkadia enhances TGF-β signalling (*12*), we hypothesized that Arkadia may affect cancer progression via enhancement of TGF-β signalling. We first examined Arkadia expression in 20 cancer cell lines. As shown in Fig. 2A, the levels of expression of Arkadia mRNA in these cell lines were not very different (3-fold at a maximum; top panel). Expression of Arkadia protein was also observed widely in these cell lines, although the levels of expression were different when the intensities of the immunoblot bands were compared (7-fold at a maximum; second panel).

We then examined the levels of expression of c-Ski and SnoN. Increased expression of c-Ski and SnoN has been reported in several human cancers (*30–32*), although in some cases this was accompanied by

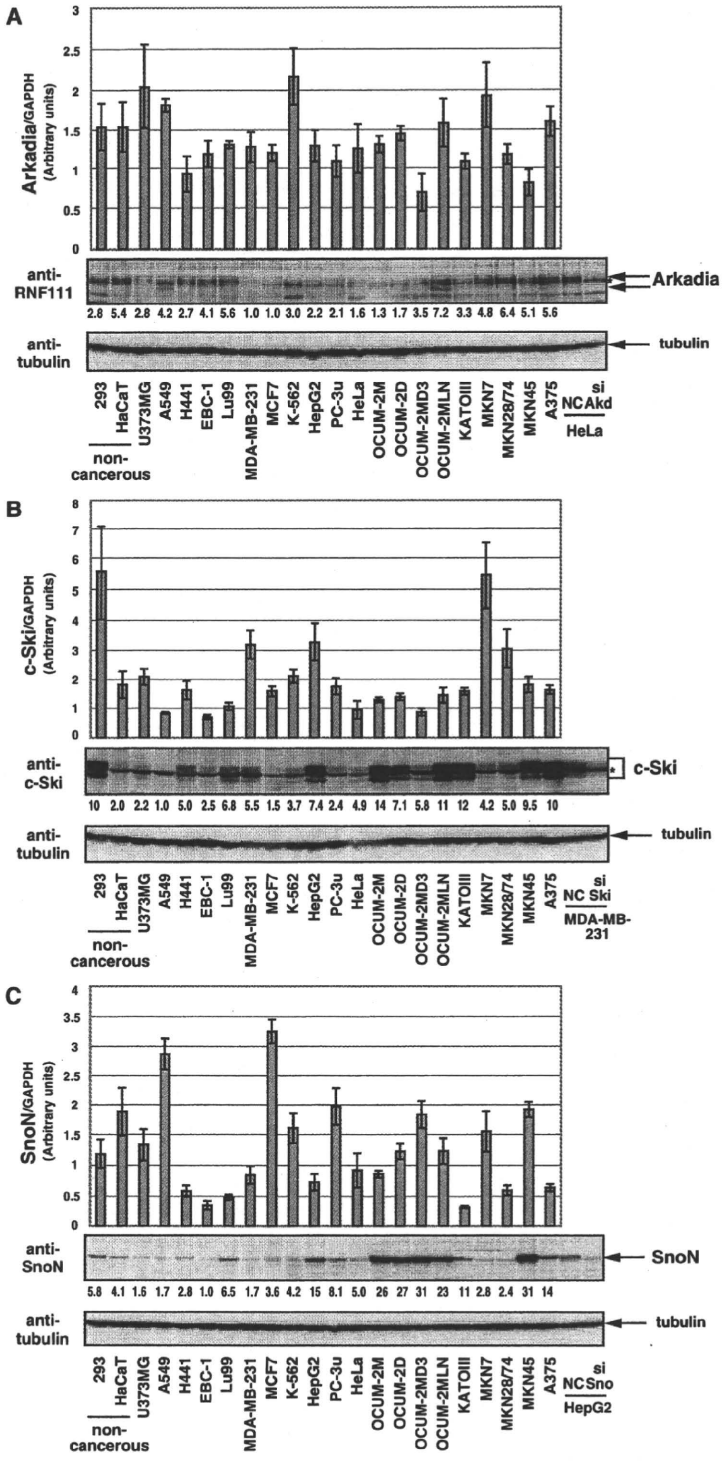

Fig. 2 mRNA and protein expressions of Arkadia, c-Ski and SnoN in various tumour cell lines. (A–C) Levels of mRNA expression of Arkadia (A), c-Ski (B) and SnoN (C) were compared among 20 tumour cell lines and two non-cancerous cell lines (top panels). Vertical axis shows relative expressions of human Arkadia (A), c-Ski (B) or SnoN (C) normalized to human GAPDH determined by real-time RT–PCR. Cell lysates were obtained in parallel with RNA preparation from the 20 tumour cell lines and two non-cancerous cell lines. The lysates were subjected to SDS–PAGE followed by immunoblotting with anti-RNF111 (A), anti-c-Ski (B) or anti-SnoN (C) (middle panels). Bands for each protein were confirmed with siRNA of Arkadia (siAkd) in HeLa cells, that of c-Ski (siSki) in MDA-MB-231 cells, and that of SnoN (siSno) in HepG2 cells (right two lanes in each panel). NC denotes negative control oligonucleotide-transfected cells. Asterisks denote non-specific bands. The specific bands of Arkadia (A), c-Ski (B) and SnoN (C) were quantified and normalized to those of tubulin. Values shown at the bottom of corresponding panels were relative to that of cells with the lowest expression of each protein.

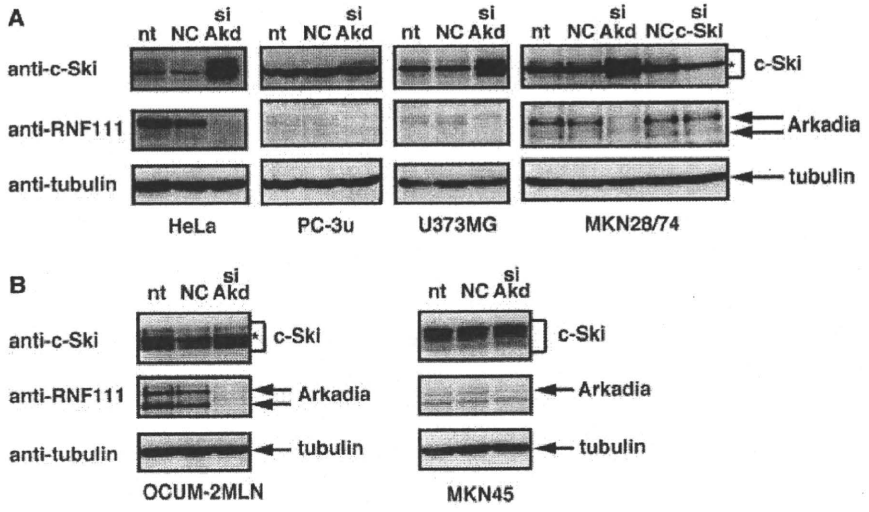

Fig. 3 Effect of knockdown of Arkadia on c-Ski protein expression. (**A** and **B**) Expression of endogenous Arkadia was knocked down by transfection of siArkadia oligonucleotide into the indicated tumour cell lines. Lysates from non-transfected cells (nt), control oligonucleotide-transfected cells (NC) and Arkadia-knocked down cells (siAkd) were subjected to SDS–PAGE followed by immunoblotting with anti-c-Ski (top panels). Bands of c-Ski were confirmed by siRNA of c-Ski in MKN28/74 cells (right two lanes in the right top panel of A). Knockdown of Arkadia was confirmed by immunoblotting with anti-RNF111 (middle panels). Asterisks denote non-specific bands.

gene amplification of c-Ski or SnoN (*29*, *33*, *34*). As shown in Fig. 2B and C, mRNA levels of c-Ski and SnoN varied markedly among the cell lines tested. Levels of c-Ski mRNA differed nearly 8-fold between EBC-1 and MKN7 (Fig. 2B, top panel), and levels of SnoN mRNA differed nearly 10-fold between KATOIII and MCF7 (Fig. 2C, top panel). Levels of expression of c-Ski/SnoN proteins were also varied markedly among these cancer cell lines. When bands of immunoblotting were quantified, the levels of expression of c-Ski protein differed 14-fold between A549 and OCUM-2M (Fig. 2B, second panel), and those of SnoN protein differed >30-fold between EBC-1 and OCUM-2MD3 (Fig. 2C, second panel).

In some cell lines, the levels of expression of c-Ski were high at the mRNA but low at the protein level (MKN7 and MKN28/74), whereas in other cell lines they were low at the mRNA level but high at the protein level (OCUM-2M, OCUM-2D, OCUM-2MLN, KATOIII, MKN45 and A375). Similar results were obtained for SnoN (Fig. 2C; A549 and MCF7).

Arkadia was thus ubiquitously expressed in various cancer cell lines at both the mRNA and protein levels, whereas in some cell lines levels of expression of c-Ski and SnoN varied at both mRNA and protein levels and levels of expression of mRNA and protein were not correlated.

Dysfunction of Arkadia in degradation of c-Ski in some cancer cell lines

Since Arkadia was expressed in all the 22 cell lines tested, we examined whether Arkadia functions as an E3 ubiquitin ligase in these cell lines. We knocked down Arkadia and determined protein expression of c-Ski. c-Ski protein accumulated in MDA-MB-231 (Fig. 1A), HeLa, PC-3u, U373MG and MKN28/74 cells upon knockdown of Arkadia (Fig. 3A), indicating that Arkadia down-regulates c-Ski in these types of cells. However, as shown in Fig. 3B, accumulation of c-Ski protein was not observed in OCUM-2MLN and MKN45 cell lines upon silencing of Arkadia. These findings suggest that Arkadia does not degrade c-Ski in some cancer cell lines. The high levels of expression of c-Ski protein in these cell lines can be attributed to dysfunction of Arkadia.

Enhancement of TGF-β target gene expression by endogenous Arkadia

We next examined mRNA expression of TGF-β target genes upon knockdown of Arkadia. HeLa cells were transfected with siRNA oligonucleotide and treated with TGF-β for the indicated periods of time. SnoN is one of the target genes of TGF-β (*42*), and, as shown in Fig. 4A, induction of SnoN mRNA was reduced (left panel) when expression of Arkadia was silenced (right panel), suggesting that endogenous Arkadia contributes to enhancement of TGF-β signalling. We also used OCUM-2MLN cells in which c-Ski protein did not accumulate upon knockdown of Arkadia. As shown in Fig. 4B, induction of target genes including *SnoN* (left top panel), *PAI-1* (left bottom panel) and *Smad7* (right bottom panel) was attenuated when Arkadia was silenced (right top panel). These findings suggest that Arkadia functions as an enhancer of TGF-β signalling in OCUM-2MLN cells, although it does not function as an E3 ubiquitin ligase for c-Ski.

Reduction of c-Ski protein expression by exogenous Arkadia

We further performed gain-of-function experiments, and examined the effects of exogenous Arkadia on c-Ski protein expression. HepG2 cells were used since the levels of expression of c-Ski and SnoN proteins

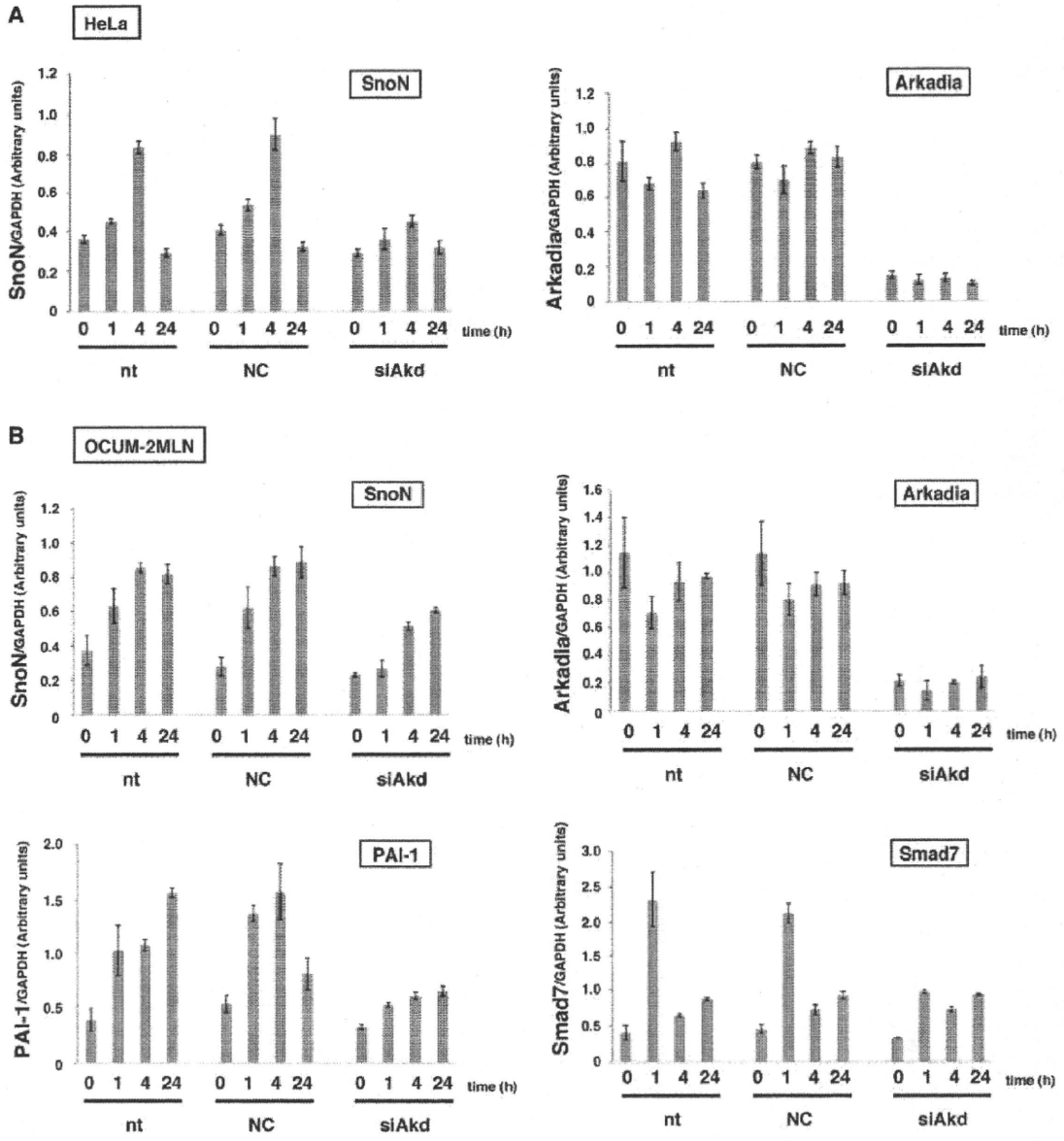

Fig. 4 Knockdown of Arkadia attenuates induction of TGF-β target genes. (**A** and **B**) HeLa cells (A) and OCUM-2MLN cells (B) were transfected with siArkadia oligonucleotide (siAkd) for silencing of endogenous Arkadia expression, or control oligonucleotide (NC), or remained untreated (nt). Cells were treated with 1 ng/ml TGF-β and harvested at the indicated time points. mRNA expression of SnoN, Arkadia, PAI-1 and Smad7 was determined by real-time RT–PCR. Vertical axis shows relative expressions of these genes normalized to human GAPDH.

were high and that of Arkadia was low in them (Fig. 2). OCUM-2MLN cells were also used since endogenous Arkadia did not contribute to degradation of endogenous c-Ski protein in them (Fig. 3B). Wild-type Arkadia (WT) or a RING finger domain-deleted mutant of Arkadia (ΔC) was expressed in these cells using a lentivirus vector expression system. Expression of Arkadia was confirmed using anti-Arkadia antibody. As shown in Fig. 5 (top panels), expression of exogenous Arkadia was higher than that of endogenous Arkadia in control cells. In both cell lines examined, c-Ski protein was down-regulated in cells expressing Arkadia-WT but up-regulated in those expressing Arkadia-ΔC. These findings showed that exogenous Arkadia induced degradation of endogenous c-Ski in these cells through its ubiquitin ligase activity.

Inhibition of growth of HepG2 cells by Arkadia in the presence and absence of TGF-β stimulation

To examine the effects of Arkadia in cancer cells, growth assay was performed using cells that express

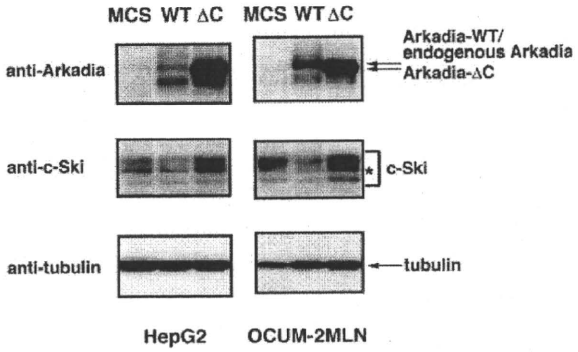

Fig. 5 Exogenous Arkadia reduces protein expression of c-Ski. HepG2 and OCUM-2MLN cells were infected with lentivirus harbouring multi-cloning site control (MCS), Arkadia-WT (WT) or Arkadia-ΔC (ΔC). Lysates from these cells were subjected to SDS–PAGE followed by immunoblotting with anti-Arkadia antibody (top panels), anti-c-Ski antibody (middle panels) and anti-tubulin antibody (bottom panels). Asterisk denotes non-specific bands.

Arkadia-WT or Arkadia-ΔC. We used HepG2 cells, since OCUM-2MLN cells do not respond to TGF-β for growth inhibition (*38*). As previously reported (*43*), growth of HepG2 cells was inhibited by treatment with TGF-β (Fig. 6A MCS). Cell growth was inhibited by expression of Arkadia-WT but not by Arkadia-ΔC in the absence of ligand. In addition, Arkadia-WT, but not Arkadia-ΔC, enhanced TGF-β-induced growth inhibition. These findings suggest that Arkadia represses HepG2 cell growth in the presence as well as the absence of TGF-β.

We then examined mRNA expression of TGF-β target genes in Arkadia-expressing HepG2 cells. Control cells (MCS), wild-type Arkadia-expressing cells (WT) and Arkadia-ΔC-expressing cells (ΔC) were treated with TGF-β for the indicated periods of time. Arkadia-WT, but not ΔC, enhanced induction of target genes of TGF-β, including *SnoN* and *Smad7* (Fig. 6B, top panels), suggesting that ectopic Arkadia enhanced TGF-β signalling through its C-terminal RING domain. Since TGF-β has been reported to

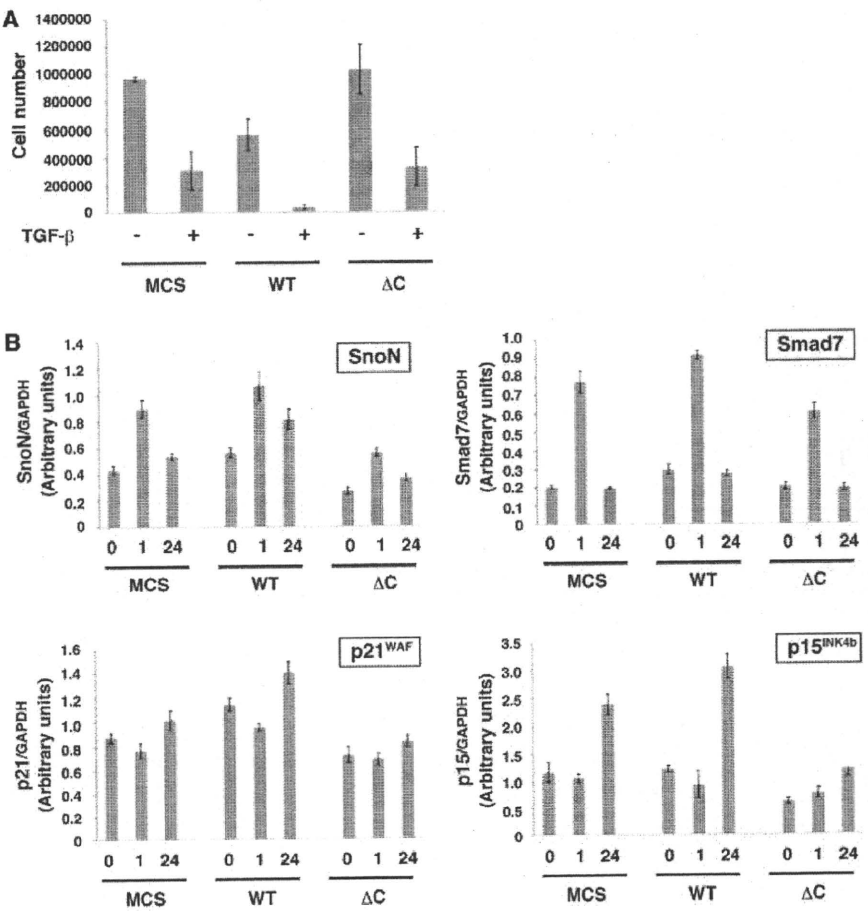

Fig. 6 Arkadia inhibits HepG2 cell growth. (**A**) Cell growth assay was performed as previously described (*43*). Numbers of HepG2 cells expressing Arkadia-WT (WT) or Arkadia-ΔC (ΔC) and control cells (MCS) were counted at day 4 with or without treatment with 0.5 ng/ml TGF-β. (**B**) HepG2 cells expressing Arkadia-WT (WT) or Arkadia-ΔC (ΔC) or control cells (MCS) were treated with 0.5 ng/ml TGF-β for the indicated periods of time. mRNA expressions of p21, p15, SnoN and Smad7 were determined by real-time RT–PCR. Vertical axis shows relative expressions of these genes normalized to human GAPDH.

inhibit cell growth by regulating expression of cell cycle regulators (*44*), we examined the expression of $p21^{WAF}$ and $p15^{INK4b}$. As shown in Fig. 6B, expression of $p21^{WAF}$ was up-regulated in WT cells but not in ΔC cells, in the presence as well as the absence of TGF-β stimulation (left bottom panel). Expression of $p15^{INK4b}$ was also up-regulated in WT cells but not in ΔC cells in the presence of TGF-β stimulation (right bottom panel). These findings suggest that Arkadia inhibits HepG2 cell growth, at least in part through induction of $p21^{WAF}$ and $p15^{INK4b}$.

Discussion

c-Ski has been reported to undergo degradation in response to TGF-β stimulation (*41*). The E3 ubiquitin ligases involved in this process have yet to be fully determined. Recently, Le Scolan *et al.* (*45*) reported that knockdown of Arkadia abrogated TGF-β-induced degradation of c-Ski, suggesting that Arkadia is responsible for the degradation of c-Ski. In our study, however, knockdown of Arkadia in MDA-MB-231 cells failed to attenuate the decrease in c-Ski protein upon TGF-β stimulation (Fig. 1A). We also found that TGF-β-stimulation resulted in down-regulation of c-Ski in $Arkadia^{-/-}$ MEFs (Fig. 1B), although the down-regulation was incomplete. These findings suggest that ubiquitin ligase(s) other than Arkadia are involved in TGF-β-induced degradation of c-Ski protein, at least under some experimental conditions. The differential effects of E3 ubiquitin ligases may depend on cell type or cellular context.

Arkadia was expressed broadly in various cancer cell lines. In contrast, the levels of expression of c-Ski/SnoN protein varied markedly among these cancer cells (Fig. 2). Interestingly, in some cancer cell lines examined, levels of expression of c-Ski/SnoN at the mRNA and protein levels were not correlated. These findings suggest that c-Ski and SnoN are regulated at the post-transcriptional level in these cancer cells. The lack of correlation between expressions of c-Ski/SnoN at the mRNA and protein levels may be due in part to dysfunction of Arkadia, since Arkadia did not degrade c-Ski protein in some of these cancer cell lines. Regulation by ubiquitin ligases other than Arkadia or regulation at translational level may also account for this lack of correlation.

In OCUM-2MLN cells, Arkadia degrades neither c-Ski protein (Fig. 3B) nor SnoN protein (our unpublished data), but does enhance TGF-β signalling (Fig. 4B). These findings indicate that endogenous Arkadia enhances TGF-β signalling through ubiquitylation of substrates other than c-Ski or SnoN. In HepG2 cells, c-Ski, SnoN and Smad7 are important substrates of Arkadia in maximal enhancement of TGF-β signalling (*13*). It remains to be determined whether Arkadia degrades Smad7 in OCUM-2MLN cells, since Smad7 protein was not detected by immunoblotting in the present study (data not shown). Thus, the possibility cannot be excluded that substrate(s) of Arkadia other than c-Ski, SnoN, or Smad7 are involved in the negative regulation of TGF-β signalling in OCUM-2MLN cells.

The question why Arkadia does not degrade c-Ski in OCUM-2MNL cells remains to be addressed. Expression of c-Ski protein was reduced when Arkadia was ectopically expressed in OCUM-2MLN cells (Fig. 5). Endogenous c-Ski in these cells is thus sensitive to degradation by Arkadia. We detected no mutations in the RING finger domain of endogenous Arkadia in OCUM-2MLN cells (data not shown), consistent with the finding that Arkadia still enhances TGF-β signalling in these cells. It is possible that in OCUM-2MLN cells Arkadia harbors mutation(s) in its c-Ski/SnoN-interacting region. Alternatively, endogenous Arkadia in these cells may be post-translationally modified and thereby lose its effects on c-Ski/SnoN. Investigation of the mutations and intracellular modifications of Arkadia is of importance for further understanding of the regulation of TGF-β signalling in cancer cells.

Overexpression of Arkadia inhibited basal growth of HepG2 cells. We found that expression of $p21^{WAF}$ was higher in HepG2 cells overexpressing Arkadia-WT than in control cells or cells overexpressing Arkadia-ΔC in the absence of TGF-β stimulation (Fig. 6B). It remains to be determined how Arkadia enhances the expression of $p21^{WAF}$ in the absence of TGF-β stimulation. Arkadia may have substrates other than c-Ski, SnoN or Smad7 when it inhibits the basal growth of HepG2 cells, although overexpression of Arkadia may exhibit non-physiological effects.

In the present study, we have shown that endogenous as well as exogenous Arkadia positively regulates the expression of TGF-β target genes in HeLa, OCUM-2MLN and HepG2 cells. In addition, we found that Arkadia inhibits the growth of HepG2 cells in the presence of TGF-β stimulation. Arkadia may function as a tumor suppressor by inhibiting the growth of tumour cells that are sensitive to TGF-β-induced cytostasis. Examination of the roles of Arkadia in late-stage cancer will also be needed in the near future. Further analysis will reveal how Arkadia regulates the dual effects of TGF-β on tumourigenesis and cancer development.

Supplementary Data

Supplementary data are available at JB online.

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Conflict of interest
None declared.

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Successful Treatment with Pemetrexed in a Patient with Mucinous Bronchioloalveolar Carcinoma

Long-Term Response Duration with Mild Toxicity

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A 36-year-old female former smoker presented with a productive cough. One year after visiting our hospital in February 2008, chest computed tomography (CT) revealed diffuse bilateral pulmonary nodules combined with a consolidation shadow (Figure 1*A*). A definite diagnosis could not be made by bronchofiberscopic examination; however, CT-guided needle biopsy specimens revealed bronchioloalveolar carcinoma (BAC) of mucinous subtype (Figure 2). Her clinical stage was T4N2M1a according to the seventh edition of the tumor, node, metastasis classification. Activating epidermal growth factor receptor (EGFR) gene mutations were not detected in her biopsy specimens. She received chemotherapy with gemcitabine and carboplatin as her first-line chemotherapy; however, her disease progressed after four cycles of chemotherapy. She then received further chemotherapy with docetaxel, erlotinib, paclitaxel, and irinotecan; however, neither regimen was effective, and her symptoms worsened. In May 2009, pemetrexed was approved for non-small cell lung cancer in Japan and was chosen as her sixth-line regimen and started in June 2009. The initial dose of pemetrexed was 500 mg/m^2 with vitamin B_{12} and folic acid supplementation. Chest CT after the two cycles of chemotherapy showed a radiographic response, and her symptoms also improved. The dose of pemetrexed was reduced to 400 mg/m^2 from the fourth cycle because of grade 3 liver dysfunction (Common Terminology Criteria for Adverse Events, version 3). Other adverse events were urticaria, skin hyperpigmentation, and general fatigue; however, they were all generally mild. Chest CT showed continuous improvement (Figure 1*B*, *C*), and her liver function has been stable since the dose reduction. Pemetrexed is currently being administered for its 20th cycle, and she is doing very well.

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DISCUSSION

BAC is a distinctive form of lung adenocarcinoma and is further divided into two subtypes: mucinous and nonmucinous.[1] Although approximately 20% of adenocarcinomas have BAC features, "pure" BAC represents less than 5% of adenocarcinomas.[2] Historically, BAC was believed to be rather refractory to cytotoxic chemotherapy, and it is still debatable whether cytotoxic chemotherapy is equally effective in BAC and other types of adenocarcinoma.[2] Recently, it became widely known that the frequency of activating mutations of EGFR, the strongest predictive factor of a response to EGFR tyrosine kinase inhibitors, such as gefitinib and erlotinib, is significantly higher in BAC[3]; however, the frequency of EGFR mutations is significantly lower in the mucinous subtype than in the nonmucinous subtype.[4]

Our patient presented with BAC of the mucinous subtype, and her tumor did not express activating EGFR mutations. She received multiple lines of chemotherapy, including platinum based, docetaxel, and erlotinib; however, only pemetrexed was effective.

Pemetrexed is a multitargeted antifolate agent and has been approved as standard first-line (combination with platinum) and second-line chemotherapy for non-small cell lung cancer, and more recently, maintenance chemotherapy with pemetrexed has been under debate. Interestingly, pemetrexed is significantly more effective for nonsquamous than squamous histology.[5] One possible explanation is that the expression of thymidylate synthase, one of the molecular targets of pemetrexed, is generally higher in squamous than nonsquamous histology; however, it needs further confirmation.

We performed immunohistochemical examination to detect the echinoderm microtubule-associated protein-like 4 gene and the anaplastic lymphoma kinase gene, fusion gene, using the intercalating antibody-enhanced polymer

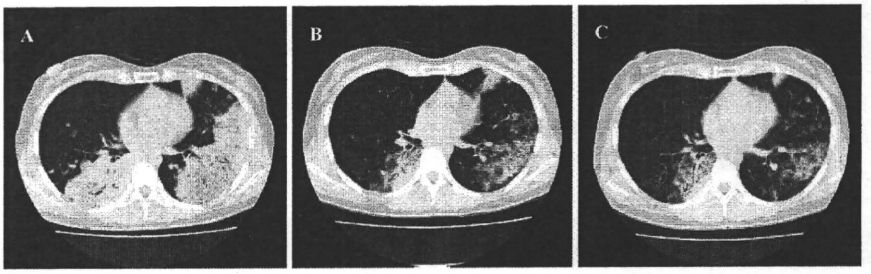

FIGURE 1. Computed tomography (CT) of the chest showed diffuse bilateral pulmonary nodules combined with consolidation shadow before pemetrexed treatment (*A*). CT after 12 cycles (*B*) and 18 cycles (*C*) showed continuous improvement of the shadow.

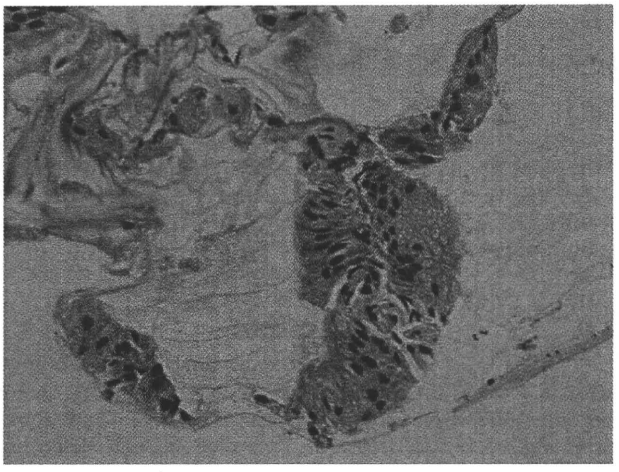

FIGURE 2. Biopsy specimen. There are a few fragments of columnar tumor cells containing mucin in the cytoplasm (hematoxylin and eosin stained).

method[6]; however, her tumor did not harbor the echinoderm microtubule-associated protein-like 4 gene and the anaplastic lymphoma kinase gene, fusion gene.

Finally, pemetrexed was safely administered for more than 1 year to our patient without deterioration of the performance status. This may also indicate the usefulness of pemetrexed as maintenance chemotherapy. Further investigations of pemetrexed are needed in patients with BAC.

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by Kengo Takeuchi, Manabu Soda, Yuki Togashi, Yasunori Ota, Yasunobu Sekiguchi, Satoko Hatano, Reimi Asaka, Masaaki Noguchi, and Hiroyuki Mano

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Identification of a novel fusion, SQSTM1-ALK, in ALK-positive large B-cell lymphoma

Running title: SQSTM1-ALK-positive large B-cell lymphoma

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