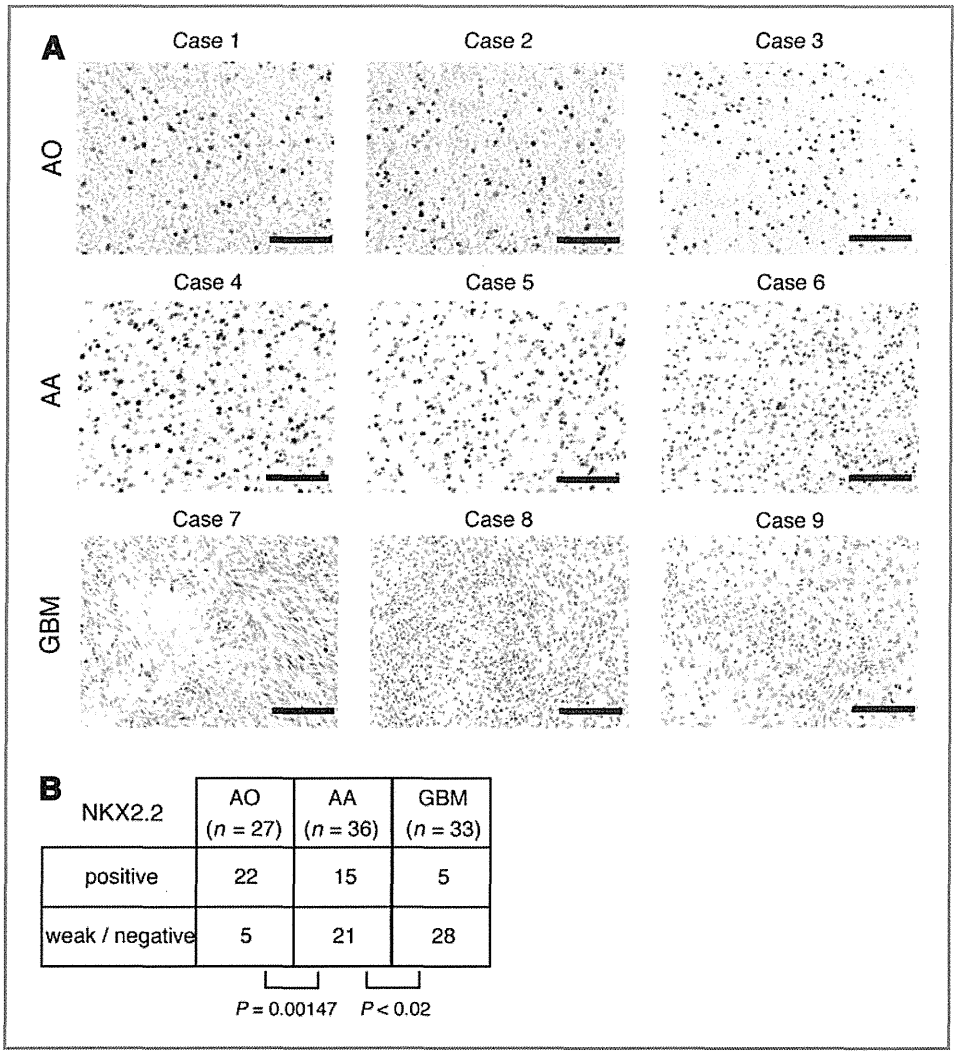

B

NKX2.2	AO (n = 27)	AA (n = 36)	GBM (n = 33)
positive	22	15	5
weak / negative	5	21	28

P = 0.00147 P < 0.02

Figure 4. Immunohistochemical examination of NKX2.2 expression in human high-grade gliomas. A, sections of 96 human gliomas were stained with anti-NKX2.2 antibody. Three representative samples of 27 AOs (cases 1–3), 36 AAs (cases 4–6), or 33 GBMs (cases 7–9) are shown. Scale bars, 100 μm. B, correlation between downregulated NKX2.2 expression and malignancy. Gliomas in (A) were scored for intensity of NKX2.2 immunostaining. The significance of the association between the level of NKX2.2 protein expression and malignancy grade was calculated, as determined by Fisher's exact test (right tail).

Discussion

Here, we have established mouse models harboring specific mutations that allow us to control stages of malignant glioma progression (AA versus GBM). These models provide significant advantages in comparing characteristics of gliomas of different malignant progression stages. Mutations seen in human cancers may differ from those seen in mouse models. Nonetheless, mouse models are essential and indispensable to fully understand the nature of gliomas. Our models represent powerful tools useful to identify novel factors that control glioma malignant progression.

A stem cell–like gene expression signature (stemness) has been shown in poorly differentiated tumors, based on histologic criteria. Stemness is associated with an unfavorable prognosis in several human cancers, including gliomas (30). Consistent with these data, we observed enhanced stemness characteristics, including upregulation of stem cell markers or sphere formation, in GBM (grade IV) tumors but not AA (grade III) in our mouse models. Although the mechanism is still unclear, several lines of evidence show that genetic loss of *p53* or $p16^{Ink4a}/p19^{Arf}$ enhances stemness. For example, *p53* or $p16^{Ink4a}/p19^{Arf}$ deficiency increases both the kinetics of induced pluripotent stem (iPS) cell reprogramming and the number of emerging iPS cell colonies (31–35). These results indicate that *p53* and $p16^{Ink4a}/p19^{Arf}$ function as barriers to cell reprogramming and acquisition of stemness. Indeed, *p53*/$p16^{Ink4a}/p19^{Arf}$–deficient multipotent hematopoietic progenitors exhibit properties of hematopoietic stem cells that can carry out long-term reconstitution of blood cells (36). Thus, *p53* and $p16^{Ink4a}/p19^{Arf}$ have a central role in limiting expansion of multipotent progenitors. Because differentiation pathways are commonly repressed in tumor cells, the above results plus our findings suggest a mechanism by which incipient neoplastic cells could gain the ability to self-renew, acquire further oncogenic mutations, and become malignant.

Our study reveals a critical role for Nkx2.2 in suppressing glioma development and GIC self-renewal. A direct effect of Nkx2.2 on oligodendroglial differentiation is supported by previous analyses of mouse spinal cord (25). Coexpression of Olig2 and Nkx2.2 in spinal cord (26) or in the ventricular and

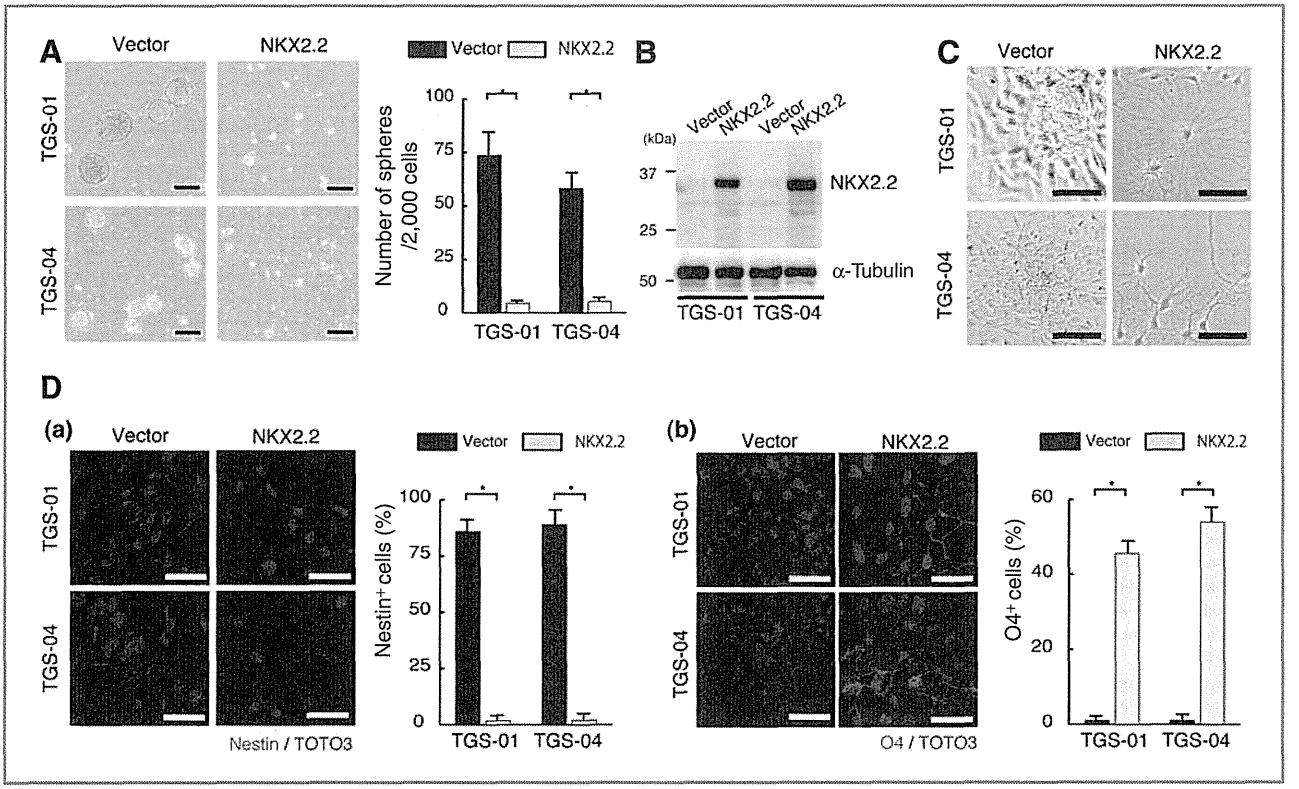

Figure 5. NKX2.2 overexpression inhibits self-renewal of human GICs by induction of oligodendroglial differentiation. A, NKX2.2 overexpression decreases human TNS formation. TGS-01 and TGS-04 cells were cultured as TNSs, transfected with pLXSB (Vector) or pLXSB-*NKX2.2* (NKX2.2), and selected with blasticidine-S for 4 days (left). Scale bars, 100 μm. Right, data shown are the mean number ± SD of TNSs generated per 2,000 cells (n = 5/group). *, $P < 0.001$. B, Western blot examination of NKX2.2 protein in representative samples from (A). α-Tubulin: loading control. C and D, decreased Nestin but increased O4 expression. TNSs were cultured on coverslips, transfected with pLXSB (Vector) or pLXSB-*NKX2.2* (NKX2.2), selected with blasticidine-S for 4 days (C, bright-field), and stained with (D, a) anti-Nestin (red) plus TOTO3, or (D, b) anti-O4 (red) plus TOTO3. Data shown represent 5 experiments. Scale bars, 50 μm. Data shown are the mean percentage ± SD of Nestin$^+$ or O4$^+$ cells among TOTO3$^+$ cells (n = 5/group). *, $P < 0.001$.

SVZ of the midbrain promotes oligodendrocyte differentiation (37). Olig2 is essential for proliferation and differentiation of oligodendrocyte precursors (38, 39). Olig2-expressing precursors give rise not only to oligodendrocytes but to motor neurons (26, 40), astrocytes, and ependymal cells (41, 42). In contrast to Olig2, Nkx2.2 generally regulates late differentiation and/or maturation, rather than initial specification, of oligodendrocyte precursors (25), although Nkx2.2 does support generation of new oligodendrocytes or remyelination in adults with CNS injury (43, 44). Nkx2.2 cooperates with Olig2 to promote oligodendrocyte maturation to Mbp-positive stages (26, 45). Although several lines of evidence suggest that Olig2 activity represents a mechanistic link between growth of malignant glioma cells and adult NPCs (46, 47), data regarding the role of Nkx2.2 role in gliomagenesis are not available. Our findings suggest that Nkx2.2 functions as a cell fate switch determining whether NPCs receiving oncogenic stimulation develop into benign glial cells or malignant astrocytomas.

Rousseau and colleagues (48) previously reported that NKX2.2 was expressed at higher levels in human AO than in AA and GBMs, suggesting that NKX2.2 is a marker that distinguishes AO from astrocytomas. In addition, it has been reported that NKX2.2 expression is elevated in a proneural subgroup of human GBM (49). In our examination of a larger group of clinically defined samples, we found that NKX2.2 is not only a marker for AO but also an indicator of malignant progression in astrocytomas, suggesting that NKX2.2 expression antagonizes malignant progression of most gliomas. In addition, we showed that impaired oligodendroglial differentiation caused by Nkx2.2 downregulation accelerates GBM formation in a robust murine model of primary gliomagenesis. Our work shows that Nkx2.2 antagonizes glioma initiation and malignant progression induced by activation of oncogenic signaling in NPCs. Finally, we show that forced Nkx2.2 expression in GICs leads to oligodendroglial differentiation and suppression of self-renewal *in vitro*. However, it is unclear whether the inhibitory effects of NKX2.2 on glioma malignancy *in vivo* are mediated by the process of oligodendroglial differentiation, because in both mouse and human samples the mature oligodendrocyte marker, MBP, is not expressed in AA. NKX2.2 may affect malignant progression by an unknown function *in vivo*. Nonetheless, reactivation of NKX2.2 expression in glioma cells suggests a novel therapeutic strategy.

In summary, our novel mouse glioma models allow us to analyze two grades of glioma rapidly and define molecular mechanisms underlying malignant glioma progression. Thus, understanding signaling driving malignant gliomagenesis in our models could contribute to development of novel approaches to diagnose and/or eradicate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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JB Commentary

Arkadia—beyond the TGF-β pathway

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Arkadia, also known as ring finger 111 (Rnf111), is an E3 ubiquitin ligase that amplifies transforming growth factor (TGF)-β family signalling through degradation of negative TGF-β signal regulators, i.e. Smad7, c-Ski and SnoN. Arkadia plays critical roles in early embryonic development through modulation of nodal signalling, as well as progression of tissue fibrosis and cancer through regulation of TGF-β signalling. Recent findings suggest that, similar to other ubiquitin ligases, including Smurf1 and 2, Arkadia regulates signalling pathways other than those of the TGF-β family. Arkadia interacts with the clathrin-adaptor 2 (AP2) complex and regulates endocytosis of certain cell surface receptors, leading to modulation of epidermal growth factor (EGF) and possibly other signalling pathways.

Keywords: TGF-β/EGF/ubiquitin ligase/AP2 complex/endocytosis.

Abbreviations: AP2, clathrin-adaptor 2; EGF, epidermal growth factor; EMT, epithelial-mesenchymal transition; R-Smads, receptor-regulated Smads; TGF-β, transforming growth factor-β.

Arkadia is an E3 ubiquitin ligase, and was originally identified as a molecule that amplifies nodal signalling. Nodal is a member of the transforming growth factor-β (TGF-β) family, which is structurally similar to activin. Formation of the node, the equivalent of *Xenopus* Spemann's organizer in mammalian embryos, plays an essential role in specification of the axis during early embryonic development. Using gene-trap mutageneis, Episkopou and colleagues (*1*, *2*) discovered Arkadia as a molecule responsible for induction of the node through enhancement of nodal signalling.

TGF-β family signalling is transduced through Smad and non-Smad pathways (*3*, *4*). Arkadia is an intracellular protein containing a RING finger domain, and has been shown to enhance TGF-β family signalling through ubiquitin-dependent degradation of some intracellular proteins. Smad7, an inhibitory Smad, suppresses TGF-β family signalling through multiple mechanisms, including physical interaction with type I receptors for TGF-β family proteins, resulting in blockade of activation of receptor-regulated Smads (R-Smads; Smad2 and Smad3 for TGF-β, activin and nodal signalling) (*4*). Arkadia physically interacts with Smad7, induces ubiquitin-dependent degradation of it, and thereby enhances TGF-β family signalling (*5*). In addition to Smad7, Arkadia has been reported to induce degradation of phospho-Smad2/3, which may lead to efficient and maximal nodal signalling for rapid resetting of target gene promoters (*6*).

In addition, TGF-β family signalling is suppressed by the transcriptional co-repressor c-Ski and its related protein SnoN in the nucleus (*4*). c-Ski and SnoN have been reported to interfere with the interaction of R-Smads with transcriptional co-activators p300 and CBP, recruit histone deacetylases to Smad complexes, and disrupt the formation of Smad complexes, leading to attenuation of TGF-β family signalling. Arkadia binds to c-Ski and SnoN, and down-regulates the levels of their expression through ubiquitin-dependent degradation (*7–9*).

Since Arkadia enhances TGF-β family signalling, it plays pivotal roles in progression of various diseases in which TGF-β family signalling is involved. TGF-β induces tissue fibrosis, suggesting that Arkadia may be involved in the pathogenesis of some fibrotic disorders. Liu *et al.* reported that levels of expression of mRNAs for type 1 collagen, TGF-β1, TGF-β type I receptor, Smad7 and Arkadia were increased in a rat model of tubulointerstitial fibrosis, while that of Smad7 protein was decreased in the kidney. They suggested that Arkadia may play a major role in degradation of Smad7, induction of epithelial-mesenchymal transition (EMT) of tubular epithelial cells, and progression of tubulointerstitial fibrosis (*10*, *11*). In support of these findings, Gai *et al.* (*12*) reported that in *Trps1* haploinsufficiency mice (TRPS1 encodes a transcription factor and is responsible for tricho-rhino-pharyngeal syndrome), tubulointerstitial fibrosis was induced by increased phosphorylation of Smad3 and decreased expression of Smad7 protein. They also found that the level of expression of Arkadia was increased in the proximal tubule cells of $Trps1^{+/-}$ mice, and suggested that Arkadia played an essential role in the induction of EMT in these cells.

The function of Arkadia may also be linked to progression of cancer. Through induction of the degradation of c-Ski, Arkadia accelerates tumor metastasis of breast and lung cancer cells in mice, possibly by induction of EMT (*9*). A study using 20 human cancer cell lines revealed ubiquitous expression of Arkadia (*13*).

Levels of expression of c-Ski and SnoN proteins varied markedly and did not correlate with those of Arkadia protein in these cells. In some cancer cell lines, including diffuse-type gastric cancer OCUM-2MLN, Arkadia failed to degrade c-Ski protein, suggesting dysfunction of it in certain cancer cells. Since TGF-β signalling interferes with the progression of diffuse-type gastric cancer OCUM-2MLN *in vivo* (*14*), perturbations of the function of Arkadia may accelerate the progression of this type of cancer.

Smurf1 and 2 were originally identified as HECT type E3 ubiquitin ligases able to induce degradation of R-Smads and suppress TGF-β family signalling. Smurf1/2 also interact with inhibitory Smads (Smad6 and 7) and degrade type I receptors for the TGF-β family proteins. Smurfs thus exhibit biological activities opposite to those of Arkadia. However, it has been found that the targets of Smurfs are not restricted to the TGF-β family signalling proteins, and that they induce degradation of many other proteins, *e.g.* Runx2, RhoA, MEKK2, Axin and p53 (*15*, *16*).

Similar to Smurf proteins, Mizutani *et al.* (*17*) found that the function of Arkadia is not limited to regulation of TGF-β family signalling. Through yeast-two-hybrid screening, the μ2 subunit of clathrin-adaptor 2 (AP2) complex was identified as an Arkadia-interacting protein. The AP2 complex plays an essential role in the endocytotic machinery that links cargo membrane proteins to the clathrin lattice (*18*). The AP2 complex is composed of four subunits, *i.e.* α, β2, μ2 and σ2. The N-terminal portion of the μ2 subunit is known to be located at the centre of the AP2 complex, while the C-terminal domain of μ2 has been shown to physically interact with Arkadia (*17*). Arkadia induced ubiquitination of the μ2 subunit, and regulated endocytosis of epidermal growth factor (EGF) receptor induced by EGF (Fig. 1). Arkadia may thus regulate a wide variety of signalling processes, in which endocytosis of the cell surface receptors is regulated by the AP2 complex. Since Arkadia-null mice are embryonic lethal, analyses of conditional knockout mice lacking expression of Arkadia in certain tissues may disclose novel *in vivo* functions of Arkadia.

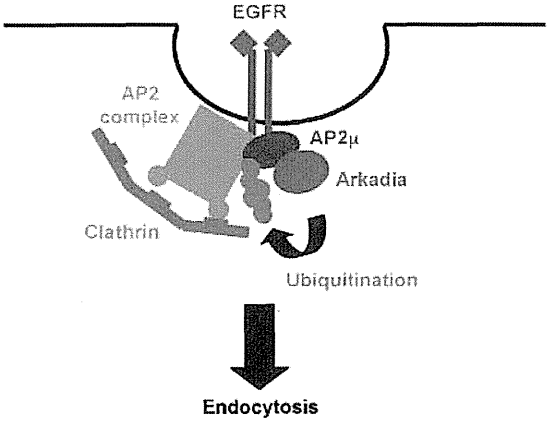

Fig. 1 Regulation of endocytosis of EGF receptor (EGFR) by Arkadia-AP2 complex. Currently, it is unknown whether AP2μ interacts with EGFR and Arkadia at the same time.

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ORIGINAL ARTICLE

Transforming growth factor-β decreases the cancer-initiating cell population within diffuse-type gastric carcinoma cells

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Stem cells in normal tissues and cancer-initiating cells (CICs) are known to be enriched in side population (SP) cells. However, the factors responsible for the regulation of expression of ABCG2, involved in efflux of dyes, in SP cells have not been fully investigated. Here, we characterized the SP cells within diffuse-type gastric carcinoma, and examined the effects of transforming growth factor-β (TGF-β) on SP cells. Diffuse-type gastric carcinoma cells established from four independent patients universally contained SP cells between 1 and 4% of total cells, which displayed greater tumorigenicity than non-SP cells did. TGF-β repressed the transcription of ABCG2 through direct binding of Smad2/3 to its promoter/enhancer, and the number of SP cells and the tumor-forming ability of cancer cells were decreased by TGF-β, although ABCG2 is not directly involved in the tumor-forming ability of SP cells. Cancer cells from metastatic site expressed much higher levels of ABCG2 and included a greater percentage of SP cells than parental cancer cells did. SP cells are thus responsible for the progression of diffuse-type gastric carcinoma, and TGF-β negatively contributes to maintain the CICs within the cancer.
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Introduction

Gastric cancer remains a major public health issue as the fourth most common cancer and the second leading cause of cancer death worldwide (Hohenberger and Gretschel, 2003; Crew and Neugut, 2006). Of the two histological subtypes of gastric cancers, the incidence of proximal, diffuse-type gastric adenocarcinoma has been increasing, particularly in Western countries. Diffuse-type gastric carcinoma is an aggressive type of gastric cancer with poor prognosis, affecting relatively young individuals. The majority of diffuse-type gastric carcinomas are in advanced stages, for which gastrectomy and chemotherapy are of limited efficacy. Thus, understanding of the mechanism underlying progression of diffuse-type gastric carcinoma is essential for management of this tumor.

The concept of 'cancer stem cells' or 'cancer-initiating cells (CICs)' is an attractive explanation for the functional heterogeneity that is commonly observed in many types of tumors. Tumors possess a hierarchical organization of cells, among which a subpopulation of stem-like cells is responsible for sustaining tumor growth (Stingl and Caldas, 2007; Visvader and Lindeman, 2008). As these cells are likely to share many of the properties of normal stem cells, which have a long lifespan, self-renewal ability, resistance to drugs, active DNA-repair activity and resistance to apoptosis, the presence of CICs is thought to be important for progression of tumors and resistance to conventional therapies (Dean *et al.*, 2005). Recently, CICs have been identified in a range of hematopoietic malignancies and solid tumors (Bonnet and Dick, 1997; Lobo *et al.*, 2007). In the maintenance of CICs, the interaction of CICs and 'niche' has critical roles, and factors secreted from the tumor microenvironment appear to regulate the maintenance of CICs in certain niches (Iwasaki and Suda, 2009). However, the factors responsible for regulation of the CIC pool have not been fully investigated.

Transforming growth factor-β (TGF-β) is the prototypic member of a family of secreted proteins that includes three isoforms of TGF-β (TGF-β1, TGF-β2 and TGF-β3), activins and bone morphogenetic proteins (BMPs). TGF-β mediates biological activities in cells through binding to heteromeric complexes of receptors on the cell surface that are composed of TGF-β type I and type II (TβRII) receptors. TGF-β type I receptor activated by TβRII phosphorylates Smad2 and Smad3, which interact with Smad4 and translocate to the

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nucleus. Nuclear Smad2/3–Smad4 complexes bind to transcription factors and transcriptional co-activators/repressors, and regulate transcription of target genes (Miyazawa *et al.*, 2002; Derynck and Zhang, 2003; Massagué, 2008). In epithelial cells, TGF-β inhibits cell proliferation, induces apoptosis and mediates differentiation, suggesting that components of the TGF-β signaling pathways have tumor-suppressive activity in epithelial tumors (Wakefield and Roberts, 2002; Bierie and Moses, 2006). Accordingly, mutations of TβRII, TGF-β type I receptor, Smad4 and Smad2, have been reported to be responsible for progression of cancers, particularly gastrointestinal tumors (Bierie and Moses, 2006).

We previously reported that deregulation of the TGF-β signaling pathway in the diffuse-type gastric carcinoma OCUM-2MLN cells, which were derived from lymph node metastasis of orthotopically implanted primary carcinoma cells, promotes the growth of primary tumors in mouse xenograft models through acceleration of angiogenesis (Kiyono *et al.*, 2009; Komuro *et al.*, 2009). In addition to this mechanism, we here present evidence that TGF-β has a novel role in regulation of the maintenance of CICs in diffuse-type gastric carcinoma cells. Our observations provide new insights into the molecular mechanisms governing TGF-β-mediated regulation of gastric carcinogenesis and may support the development of new strategies for the treatment of diffuse-type gastric carcinoma.

Results

CICs are enriched in 2MLN-dnTβRII cells

Tumor growth of diffuse-type gastric carcinoma OCUM-2MLN cells is reported to be enhanced by inhibition of the TGF-β signaling pathway in cancer cells (Kiyono *et al.*, 2009; Komuro *et al.*, 2009). In accordance with these reports, TGF-β-induced phosphorylation of Smad2 was completely inhibited in OCUM-2MLN cells, which stably expressed dominant negative form of TβRII (2MLN-dnTβRII), but not in control cells, which expressed green fluorescent protein (2MLN-GFP) (Figure 1a). TGF-β inhibited the proliferation of keratinocyte HaCaT cells *in vitro*, whereas TGF-β did not affect the rate of growth of the OCUM-2MLN cells (Figure 1b). Cells enter the S phase and proliferate when the phosphorylated retinoblastoma protein (RB) is hyperphosphorylated, whereas cells are arrested in G1 when phosphorylated RB is hypophosphorylated (Weinberg, 1995). Treatment of HaCaT cells with TGF-β decreased hyperphosphorylated RB expression, whereas hyperphosphorylated RB expression in OCUM-2MLN cells was not altered by TGF-β (Figure 1c). In contrast to *in vitro* cell proliferation, *in vivo* tumor growth of 2MLN-dnTβRII cells was accelerated in the subcutaneous mouse tumor model compared with that of 2MLN-GFP cells (Figure 1d). In the previous study, we showed that angiogenesis was accelerated in 2MLN-dnTβRII tumors through repression of the expression of the anti-angiogenic factor thrombospondin-1 (Komuro *et al.*, 2009). However, thrombospondin-1 expression is not sufficient to account for all the histological change in tumor obtained by the overexpression of dnTβRII, such as the accumulation of fibrotic tissues. Thus, we attempted here to uncover additional underlying mechanisms by which the deregulation of TGF-β signaling in OCUM-2MLN cells promotes *in vivo* tumor growth, and especially focused on the heterogeneity of cancer cells.

We examined whether 2MLN-dnTβRII cells include a higher proportion of CICs than 2MLN-GFP cells do. Various amounts of 2MLN-GFP and 2MLN-dnTβRII cells were injected into nude mice and compared for tumor-forming ability (Figure 1e). In 2MLN-dnTβRII cells, subcutaneous tumor formation required injection of 1×10^3 cells, whereas injection of 3×10^3 2MLN-GFP cells constantly failed to induce tumor formation in all mice examined ($n=4$). These findings suggested that 2MLN-dnTβRII cells promote tumor formation through enrichment of CICs in cancer cells.

ABCG2 is highly expressed in 2MLN-dnTβRII tumors

We hypothesized that TGF-β from the tumor microenvironment decreases the tumor-forming ability of OCUM-2MLN cells *in vivo*. To examine the alterations caused by disruption of the TGF-β signaling pathway in diffuse-type gastric carcinoma cells *in vivo*, we made use of comprehensive gene expression data sets for these tumors previously obtained using oligonucleotide microarray analysis (Komuro *et al.*, 2009). In our previous study, DAVID functional annotation clustering revealed that the expression of thrombospondin-1 in tumor tissues was increased by TGF-β, and thrombospondin-1 was reported as one of the 'TGF-β-positively regulated genes' in OCUM-2MLN cells (Dennis *et al.*, 2003). However, novel candidate gene(s) related to the 'tumor-forming ability' or 'stemness' of 2MLN-dnTβRII cells was not picked up among these 'TGF-β-positively regulated genes'. Thus, genes that were strongly expressed in 2MLN-dnTβRII tumors, namely 'TGF-β-negatively regulated genes', were focused, and identified as shown in Figure 2a.

Among the 30 genes upregulated in 2MLN-dnTβRII tumors, ATP-binding cassette G2 (ABCG2) was included (Figure 2a). ABCG2 contributes to efflux of many types of dyes, including Hoechst 33342. Although uptake of Hoechst 33342 occurs universally in all cells, efflux of it is less common. Cells with efflux capacity conferred by ABCG2 expression are negatively stained on flow cytometric analysis, and referred to as side population (SP) cells on dot plots. As ABCG2 expression and SP cells are thought to be of importance in the isolation of CICs (Wu and Alman, 2008), we focused on the function of ABCG2.

TGF-β regulates the transcription of ABCG2 through the Smad pathway

First, we examined whether ABCG2 is one of the downstream targets of TGF-β in OCUM-2MLN cells by quantitative real-time reverse transcription–PCR

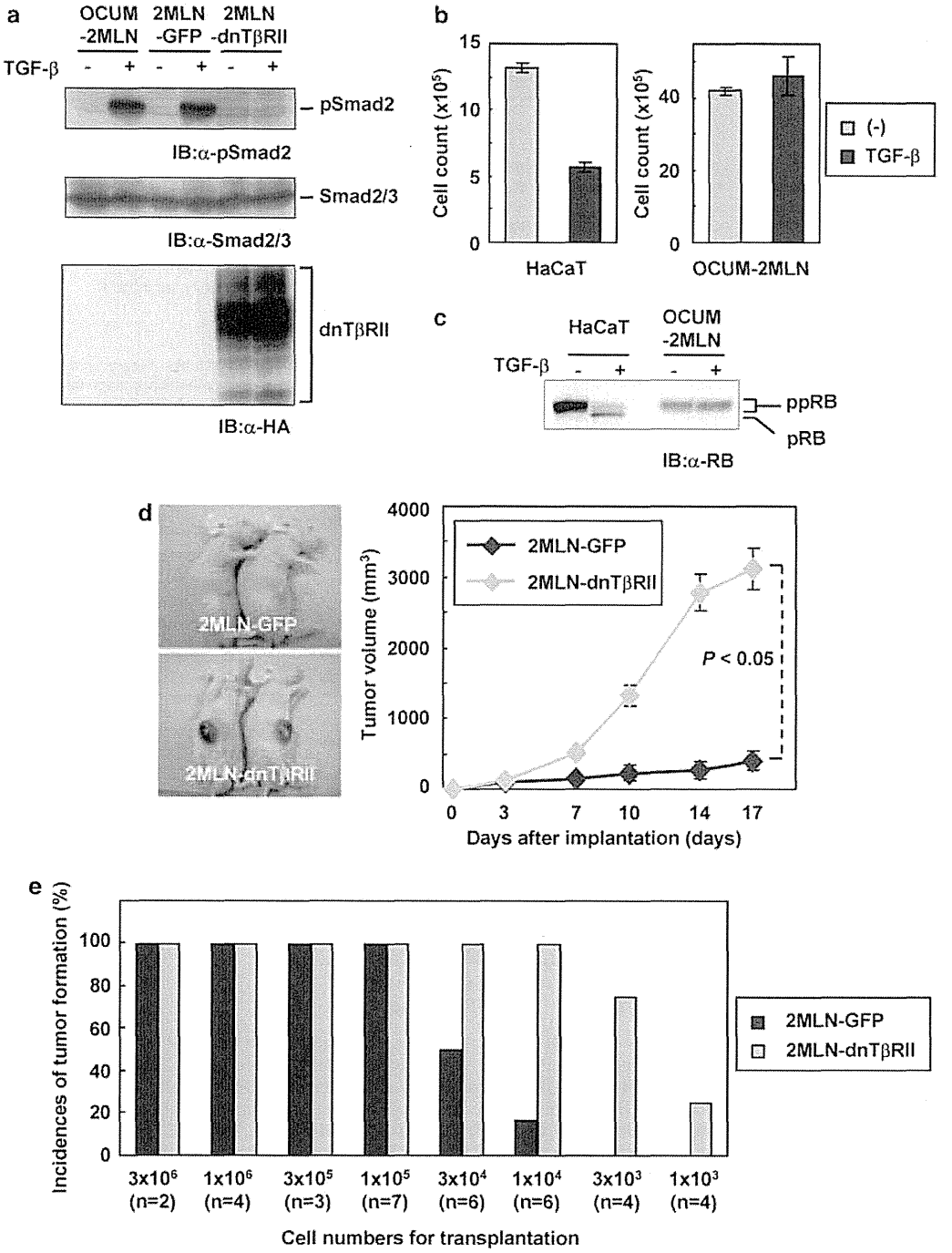
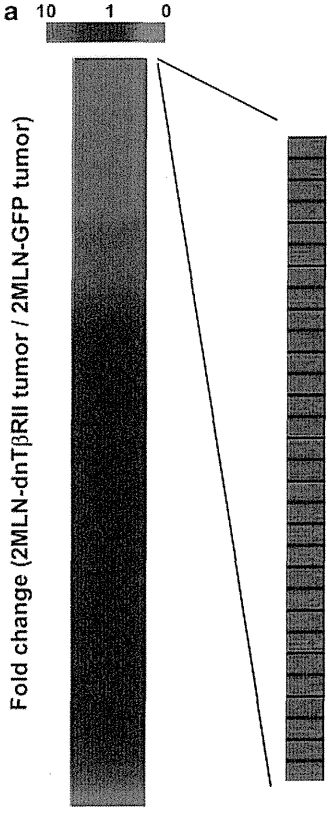


Figure 1 CICs are enriched in 2MLN-dnTβRII cells. (**a**) OCUM-2MLN cells were infected with lentiviruses carrying GFP cDNA (2MLN-GFP) and dnTβRII cDNA (2MLN-dnTβRII), and treated with TGF-β (1 ng/ml) for 1 h. Cell lysates were subjected to immunoblotting with indicated antibodies. (**b**) HaCaT and OCUM-2MLN cells were treated with TGF-β (1 ng/ml) for 4 days, and counted. *Columns*, mean of duplicate determinations; *bars*, s.d. (**c**) Hyperphosphorylation of pRB in the cells in (**b**) was determined with anti-phospho-pRB antibody. RB proteins migrate as multiple bands because of varying degrees of phosphorylation. (**d**) A total of 3×10^6 OCUM-2MLN or 2MLN-dnTβRII cells were xenografted. Representative photographs (*left panels*) and tumor volumes (*right panel*) were indicated. *Points*, mean; *bars*, s.d. (**e**) The indicated numbers of 2MLN-GFP or 2MLN-dnTβRII cells were injected into BALB/c *nu/nu* mice. Incidences of tumor formation 4 weeks after injection are shown.

(RT–PCR) (Figure 2b). Expression of ABCG2 was downregulated in response to exogenous TGF-β in parental OCUM-2MLN and 2MLN-GFP cells, whereas similar repression was not apparent in 2MLN-dnTβRII cells. We also demonstrated that treatment with TGF-β, but not with BMP-4, downregulated the *ABCG2* messenger RNA (Figure 3a) and ABCG2 protein (Figure 3b) in OCUM-2MLN cells.

In order to confirm the involvement of the Smad-dependent signal transduction by TGF-β, we knocked



Fold change	Probe sets	Gene symbol	Gene title
10.04	228607_at	OAS2	2'-5'-oligoadenylate synthetase 2, 69/71kDa
7.78	205869_at	PRSS1	protease, serine, 1 (trypsin 1)
7.25	216387_x_at	LOC390411	nucleolar phosphoprotein B23
7.09	204439_at	IFI44L	interferon-induced protein 44-like
6.56	1553033_at	SYTL5	synaptotagmin-like 5
5.53	203535_at	S100A9	S100 calcium binding protein A9 (calgranulin B)
4.48	1554436_a_at	REG4	regenerating islet-derived family, member 4
4.39	214079_at	DHRS2	dehydrogenase/reductase (SDR family) member 2
4.21	206561_s_at	AKR1B10	aldo-keto reductase family 1, member B10
4.21	223447_at	REG4	regenerating islet-derived family, member 4
4.01	230765_at	KIAA1239	KIAA1239 protein
3.87	208170_s_at	TRIM31	tripartite motif-containing 31
3.86	210143_at	ANXA10	annexin A10
3.85	204272_at	LGALS4	lectin, galactoside-binding, soluble, 4 (galectin 4)
3.83	204475_at	MMP1	matrix metalloproteinase 1 (interstitial collagenase)
3.77	202388_at	RGS2	regulator of G-protein signalling 2, 24kDa
3.77	203058_s_at	PAPSS2	3'-phosphoadenosine 5'-phosphosulfate synthase 2
3.69	219140_s_at	RBP4	retinol binding protein 4, plasma
3.66	203717_at	DPP4	dipeptidylpeptidase 4 (CD26)
3.64	242093_at	---	---
3.63	225207_at	---	---
3.52	209735_at	ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2
3.45	221577_x_at	GDF15	growth differentiation factor 15
3.44	203691_at	PI3	protease inhibitor 3, skin-derived (SKALP)
3.40	204972_at	OAS2	2'-5'-oligoadenylate synthetase 2, 69/71kDa
3.37	206155_at	ABCC2	ATP-binding cassette, sub-family C (CFTR/MRP), member 2
3.34	226757_at	IFIT2	interferon-induced protein with tetratricopeptide repeats 2
3.30	207826_s_at	ID3	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein
3.19	220104_at	ZC3HAV1	zinc finger CCCH type, antiviral 1
3.07	204994_at	MX2	myxovirus (influenza virus) resistance 2 (mouse)

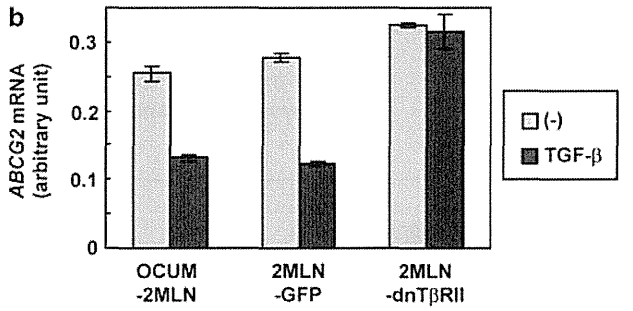


Figure 2 *ABCG2* is highly expressed in 2MLN-dnTβRII tumors. (**a**) Gene expression in 2MLN-GFP and 2MLN-dnTβRII tumors was analyzed on the GeneChip Human Genome U133 Plus 2.0 Array. Genes strongly expressed in 2MLN-dnTβRII tumors were purified as follows: (1) Signal intensities in 2MLN-dnTβRII were >100, and given 'PRESENT' calls: 13208 probe sets met this restriction. (2) Signals intensities in 2MLN-dnTβRII were increased more than threefold, compared with signal intensities in 2MLN-GFP: 30 of the 13208 probe sets met this restriction, all of which are listed. (**b**) OCUM-2MLN, 2MLN-GFP and 2MLN-dnTβRII cells were treated with TGF-β (1 ng/ml) for 24 h, and expression of *ABCG2* mRNA was examined by quantitative real-time RT–PCR. *Columns*, mean; *bars*, s.d.

down the endogenous expression of Smad4. Smad4 expression in OCUM-2MLN cells was successfully silenced by small interfering RNA (siRNA) targeting Smad4 (Figure 3c), and TGF-β-induced ABCG2 reduction was abolished by Smad4 knockdown, indicating that TGF-β regulates the expression of ABCG2 through the Smad-dependent pathway. Though downregulation of *ABCG2* messenger RNA by TGF-β in OCUM-2MLN cells was augmented at 8 h treatment with TGF-β, slight decrease in the expression of *ABCG2* messenger RNA was already observed only after the first 1 h (Figure 3a). Furthermore, the decrease in expression of ABCG2 by TGF-β was not abolished by suppression of *de novo* protein synthesis by treatment of OCUM-2MLN cells with cycloheximide (Figure 3d). To determine whether ABCG2 is a direct target of TGF-β in OCUM-2MLN cells, we performed chromatin immunoprecipitation using antibody against Smad2/3, DNA-binding mediators of Smad-dependent signal transduction. We have demonstrated that activated Smad2/3 directly bound to the promoter elements located in 200 bp upstream of the start point of transcription of *ABCG2* and also bound to the enhancer element located in the first intron of *ABCG2* in response

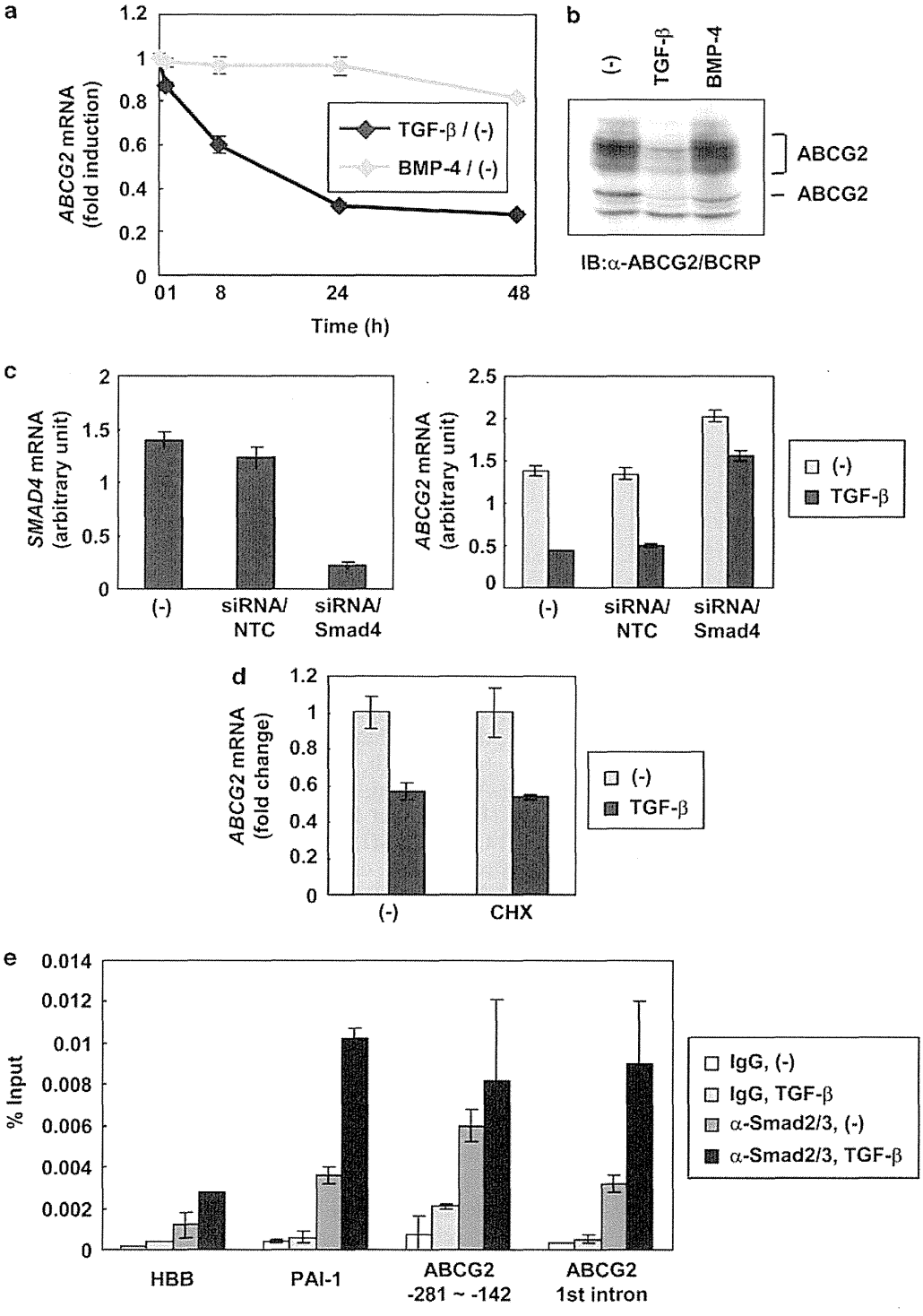


Figure 3 ABCG2 is a direct target of TGF-β. (**a**) OCUM-2MLN cells were treated with TGF-β (1 ng/ml) or BMP-4 (30 ng/ml). Expression of *ABCG2* mRNA at the indicated time points was examined by quantitative real-time RT–PCR. Fold-induction by TGF-β or BMP-4 stimulation is indicated. *Points*, mean. (**b**) OCUM-2MLN cells were stimulated with TGF-β (1 ng/ml) or BMP-4 (30 ng/ml) for 72 h, and expression of ABCG2 protein was examined by immunoblotting using anti-ABCG2/BCRB antibody. (**c**) OCUM-2MLN cells were transfected in the presence of either siRNA/Smad4 or control siRNA/NTC. At 9 h after transfection, cells were treated with TGF-β (1 ng/ml) for further 40 h. Levels of expression of *SMAD4* mRNA and *ABCG2* mRNA were determined by quantitative real-time RT–PCR. *Columns*, mean; *bars*, s.d. (**d**) OCUM-2MLN cells were treated with TGF-β (1 ng/ml) in the absence or presence of cycloheximide (CHX) (1 μg/ml). Total RNA was extracted 24 h after stimulation, and expression of *ABCG2* mRNA were examined by quantitative real-time RT–PCR. Fold-induction by TGF-β stimulation is indicated. *Columns*, mean; *bars*, s.d. (**e**) Association of the Smad complex with the ABCG2 promoter/enhancer elements. OCUM-2MLN cells were treated with TGF-β (1 ng/ml). Chromatin immunoprecipitation using anti-Smad2/3 antibody was preformed. Smad2/3-bound DNAs at 1.5 h after TGF-β treatment were examined by quantitative real-time RT–PCR using indicated primers. *Columns*, mean; *bars*, s.d.

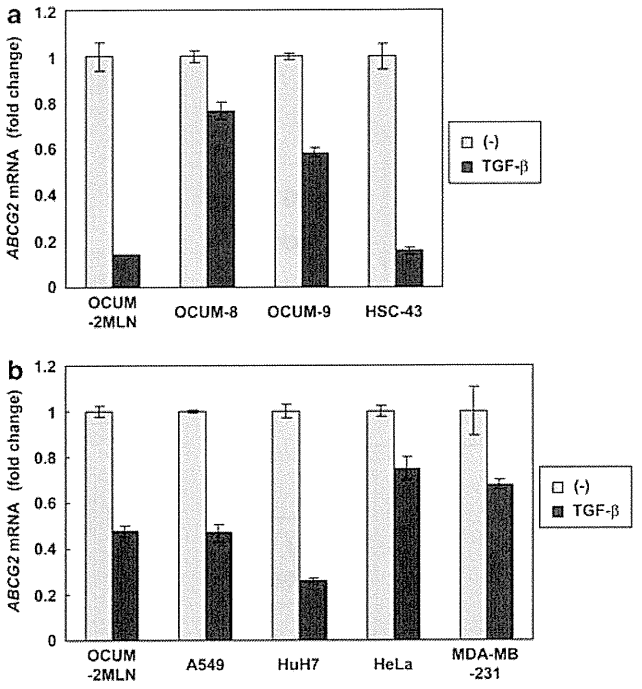


Figure 4 TGF-β regulates the transcription of ABCG2 in various cancers. (**a**) Diffuse-type gastric carcinoma cells were treated with TGF-β (1 ng/ml) for 72 h. Expression of *ABCG2* mRNA was examined by quantitative real-time RT–PCR. Fold-induction by TGF-β stimulation is indicated. *Columns*, mean; *bars*, s.d. (**b**) The indicated cancer cells were treated with TGF-β (1 ng/ml) for 72 h. Expression of *ABCG2* mRNA was examined by quantitative real-time RT–PCR. Fold-induction by TGF-β stimulation is indicated. *Columns*, mean; *bars*, s.d.

to TGF-β (Figure 3e). Taken together, these findings indicate that ABCG2 is a direct target gene of TGF-β signaling.

Transcriptional regulation of ABCG2 by TGF-β was also examined using other diffuse-type gastric carcinoma cells established form other patients, including OCUM-8, OCUM-9 and HSC-43 cells (Figure 4a). Quantitative real-time RT–PCR revealed decrease in ABCG2 expression by TGF-β in these cells, although degrees of the decrease in ABCG2 expression were variable. Moreover, similar to OCUM-2MLN cells, downregulation of ABCG2 by exogenous TGF-β was commonly observed in neoplasm arising in other organs, including lung, liver, uterus and mammary glands (Figure 4b).

SP cells display tumorigenic property

To confirm whether ABCG2 transporter was functional in OCUM-2MLN cells, we tested for the presence of SP cells in OCUM-2MLN cells by staining with Hoechst 33342. OCUM-2MLN cells included a distinct fraction of SP cells, comprising 1.42% of the total cell population, which was decreased by pretreatment with transporter inhibitors, verapamil, reserpine or fumitremorgin C (Figure 5a). Among many types of ABC transporters, ABCG2 activity is known to be completely inhibited by fumitremorgin C (Rabindran *et al.*, 2000), indicating that ABCG2 expressed in SP cells is critical for the efflux of Hoechst 33342. In agreement with this finding, knockdown of ABCG2 expression in OCUM-2MLN cells resulted in the decrease in the number of SP cells (data not shown).

Next, to examine the characteristics of SP cells, we sorted the SP cells (Figure 5b) and cultured *in vitro* for 7 days. Then, cells were re-stained with Hoechst 33342 and subjected to flow cytometric analysis. SP cells reproduced both SP cells and non-SP cells, indicating that the SP cells within OCUM-2MLN cells had repopulating capacity.

To directly determine whether CICs are enriched in SP cells *in vivo*, equal numbers of SP cells or non-SP cells were transplanted into nude mice. Tumor volume of SP cell-xenografted mice was statistically significantly larger than that of non-SP cell-xenografted mice (Figure 5c). These findings suggested that the OCUM-2MLN cells consist of heterogeneous cell populations, and that SP cells have a more important role than non-SP cells in the tumor formation by OCUM-2MLN cells as 'CICs' within cancer tissues.

TGF-β diminishes the SP fraction of OCUM-2MLN cells

As SP cells appear to be of crucial importance for tumor formation *in vivo*, we hypothesized that TGF-β diminishes the SP fraction of OCUM-2MLN cells through repression of ABCG2 transcription. To test this hypothesis, we prepared OCUM-2MLN cells pretreated with TGF-β or BMP-4 and characterized them. As expected, flow cytometric analysis demonstrated that TGF-β, but not BMP-4, diminished the SP fraction of OCUM-2MLN cells (Figure 6a). TGF-β-mediated regulation of SP cells was also observed in other diffuse-type gastric carcinoma cells (Figure 6b). These results were correlated to the expression of ABCG2 (Figures 3a and 4a).

Next, we examined whether TGF-β decreases the tumor-forming potential of OCUM-2MLN cells *in vitro* and *in vivo*. Colony formation of OCUM-2MLN cells was inhibited by treatment with TGF-β (Figure 6c). In a mouse xenograft experiment, subcutaneous tumor was formed by the injection of 1×10^5 control cells, while TGF-β-treated cells exhibited limited tumorigenesity even if the same amounts of control cells were injected (Figure 6d). These findings suggested that TGF-β decreased the SP fraction of OCUM-2MLN cells, and thereby attenuated the tumor-forming ability of OCUM-2MLN cells.

Silencing of endogenous ABCG2 does not affect the tumorigenesis of OCUM-2MLN cells

Next we examined whether the ABCG2 expression is functionally linked to the tumorigenic activity of OCUM-2MLN cells. We established the cells termed 2MLN-shRNA/ABCG2, whose expression of ABCG2 was stably knocked down by the transfer of short hairpin RNA (Supplementary Figure S1A). Though successful silencing of endogenous ABCG2 expression was achieved, 2MLN-shRNA/ABCG2 cells exhibited a

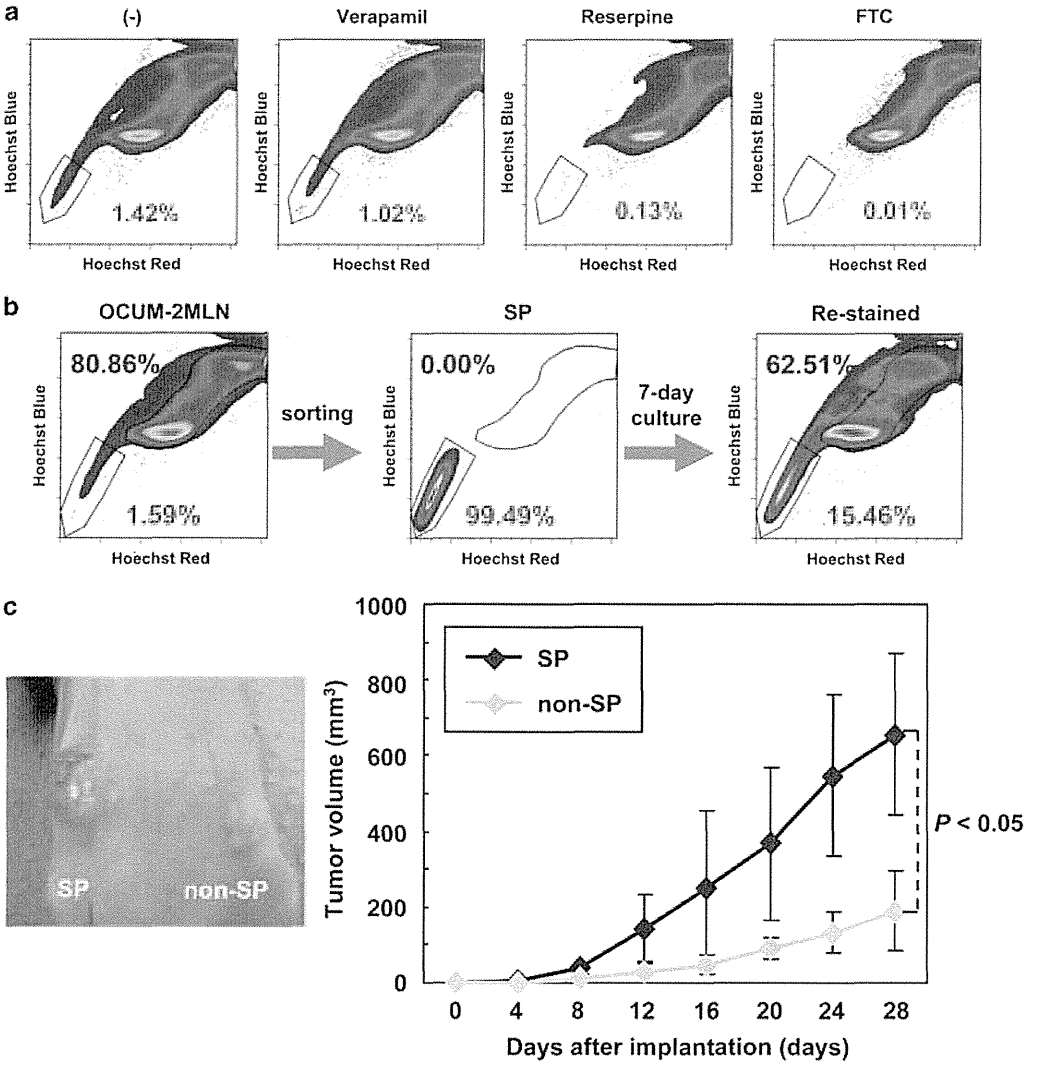


Figure 5 SP cells display tumorigenicity. (**a**) OCUM-2MLN cells were stained with Hoechst 33342 in the absence or presence of verapamil, reserpine or fumitremorgin C (FTC), and analyzed by flow cytometry. *Red numbers*, SP percentage of entire viable cell population. (**b**) SP cells were sorted from OCUM-2MLN cells. After 7-day *in vitro* culture of the separated cells, cells were re-stained with Hoechst 33342 and analyzed by flow cytometry. *Red numbers*, SP percentage of entire viable cell population; *Blue numbers*, non-SP percentage of entire viable cell population. (**c**) SP cells and non-SP cells were sorted from OCUM-2MLN cells separately. A total of 5×10^4 SP or non-SP cells were xenografted into BALB/c *nu/nu* mice. Representative photographs (*left panel*) and tumor volumes (*right panel*) were indicated. *Points*, mean; *bars*, s.d.

similar tumor forming ability to control 2MLN-shRNA/NTC cells *in vivo* (Supplementary Figure S1B). Thus, the importance of ABCG2 expression in cancer cells was not demonstrated by 'loss-of-function analysis', although this finding is consistent with previous reports, showing that ABCG2$^+$ and ABCG2$^-$ cancer cells exhibited similar tumorigenic abilities (Patrawala *et al.*, 2005).

Cancer cells from metastatic sites include more SP cells than those from primary sites

Finally, we addressed the importance of SP cells in progression of diffuse-type gastric carcinoma using a series of gastric carcinoma cell variants from the same patient (Yashiro *et al.*, 1994, 1996; Inoue *et al.*, 1997; Fujihara *et al.*, 1998): OCUM-2M (primary tumor), OCUM-2MLN (lymph node metastasis of orthotopically implanted OCUM-2M cells), OCUM-2MD3 (peritoneal metastasis of orthotopically implanted OCUM-2M cells) and OCUM-2D (pleural effusion of the same patient as yielded OCUM-2M cells) (Figure 7a). Flow cytometric analysis revealed that OCUM-2M, OCUM-2MLN, OCUM-2MD3 and OCUM-2D cells included SP cells at 0.00, 1.54, 0.14, and 0.04% of total cell populations, respectively (Figure 7b). Consistent with these findings, expression of ABCG2 in OCUM-2MLN cells was significantly higher than that in the other types of gastric carcinoma cells, and that TGF-β suppressed the expression of *ABCG2* messenger RNA (Figure 7c). In a mouse xenograft experiment, only OCUM-2MLN cells, and

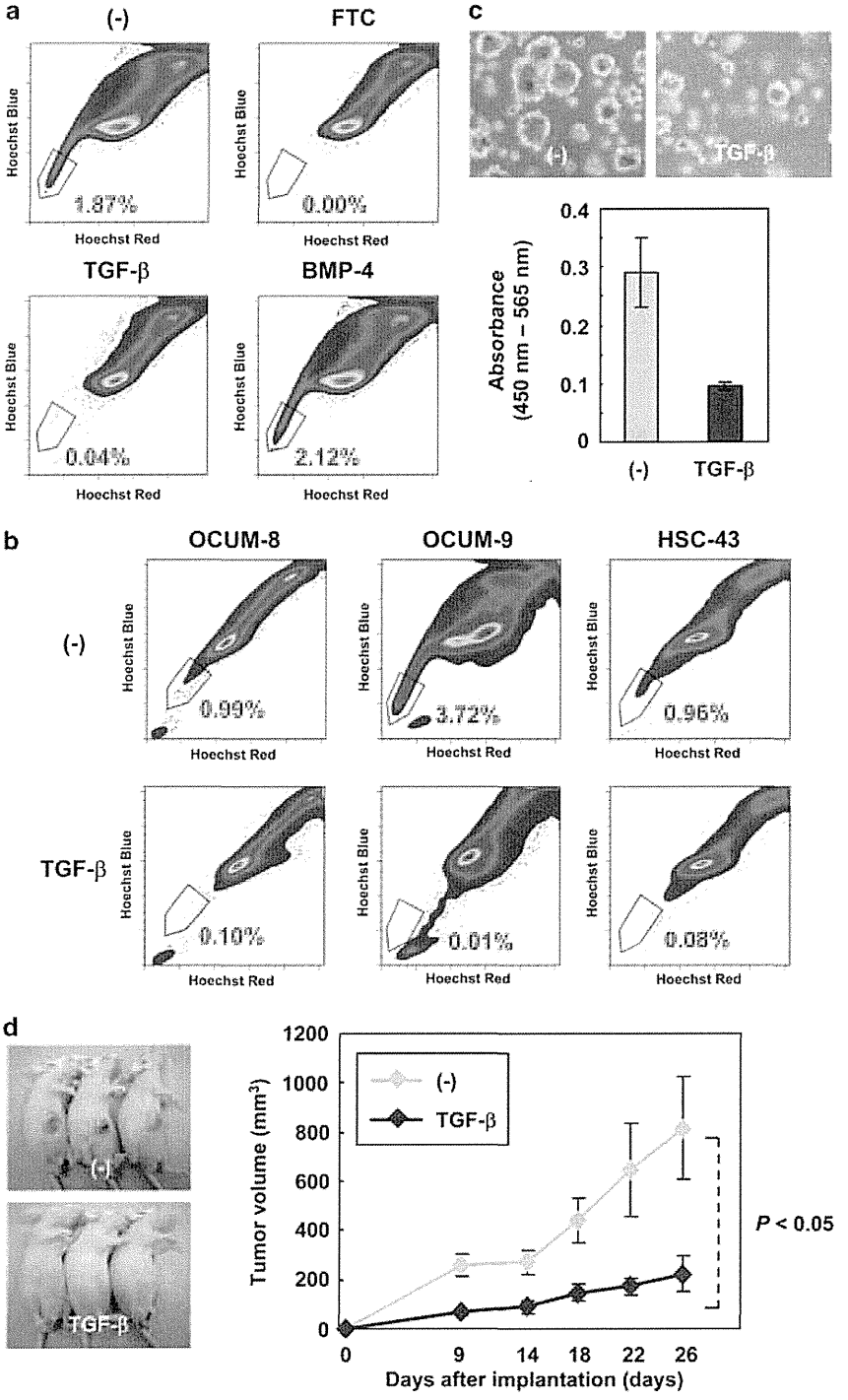


Figure 6 TGF-β decreases the SP fraction of OCUM-2MLN cells. (**a**) OCUM-2MLN cells were treated with TGF-β (1 ng/ml) or BMP-4 (30 ng/ml) for 72 h. Cells were stained with Hoechst 33342 and analyzed by flow cytometry. *Red numbers*, SP percentage of entire viable cell population. (**b**) Diffuse-type 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.

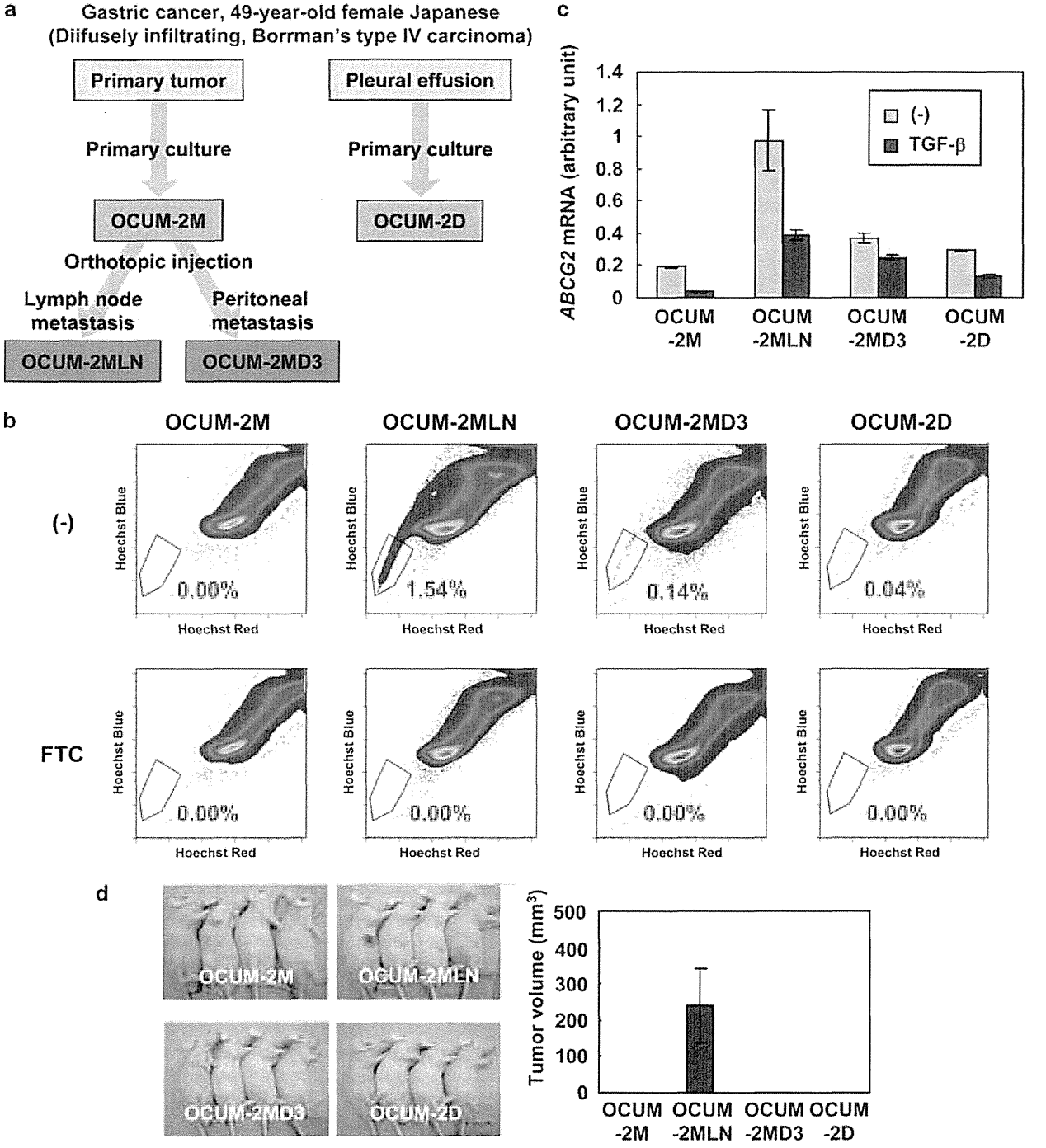


Figure 7 Highly metastatic cancer cells include more SP fractions. (**a**) Schematic representation of the *in vivo* process of selection of diffuse-type gastric carcinoma cells. (**b**) Diffuse-type gastric carcinoma cells were stained with Hoechst 33342 in the absence or presence of fumitremorgin C (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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