TABLE 2. *Correlation of Rsf-1 expression with clinical characteristics in ovarian clear cell carcinomas*

	Rsf-1 (HBXAP) expression		
Clinical characteristics	Positive	Negative	*P*
Age (n = 89)			
≥50	46 (79%)	12 (21%)	
<50	27 (87%)	4 (13%)	0.36
Stage (n = 67)			
I, II	34 (72%)	13 (28%)	
III, IV	20 (100%)	0 (0%)	0.0088*
Peritoneal dissemination (n = 79)			
Negative	53 (80%)	13 (20%)	
Positive	12 (92%)	1 (8%)	0.30
Lymph node metastasis (n = 70)			
Negative	40 (73%)	15 (27%)	
Positive	15 (100%)	0 (0%)	0.023*
Survival status (n = 89)			
Alive	58 (81%)	14 (19%)	
Deceased	15 (88%)	2 (12%)	0.46

* Statistically significant.

between tumor stage and overall survival in the CCCs, and observed that the stage III/IV cases (n = 20) had a poorer prognosis than the stage I/II cases (n = 47) ($P < 0.0001$) (Fig. 3). However, the Kaplan-Meier analysis did not show a significant difference in survival between the Rsf-1-positive and negative cases ($P = 0.42$).

DISCUSSION

An increase in the DNA copy number at the chromosome 11q13.5 locus containing Rsf-1 (HBXAP) is detected in several types of human cancers including ovarian high-grade serous carcinoma. Rsf-1 (HBXAP) encodes for a cellular nuclear protein that binds to hSNF2H (18), forming a chromatin-remodeling protein complex called RSF (remodeling and spacing factor) (19,20). Rsf-1 (HBXAP) has been shown to function as a histone chaperone in the nuclei, whereas its binding partner, hSNF2H, possesses nucleosome-dependent ATPase activity (21). The Rsf-1/hSNF2H complex (RSF complex) mediates ATP-dependent chromatin remodeling, which alters the chromatin structure or positioning of nucleosomes (20). At the cellular level, RSF participates in chromatin remodeling in response to a variety of growth signals and environmental cues. Such nucleosome remodeling is required for transcriptional activation or repression (22–24), DNA replication (25), and cell cycle progression (26).

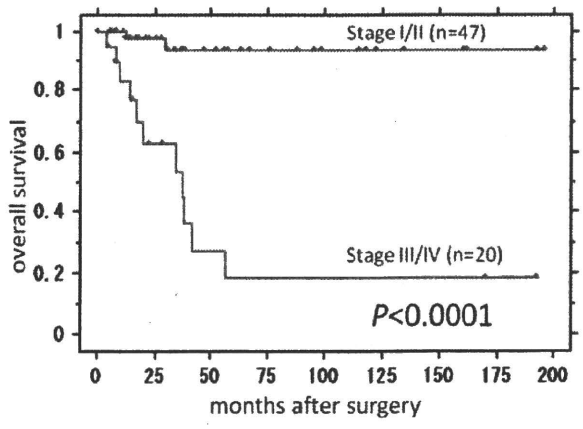

FIG. 3. Kaplan-Meier survival curve analysis shows that patients with stage III/IV ovarian clear cell carcinoma have a significantly worse overall survival rate than those with stage I/II ovarian clear cell carcinoma ($P < 0.0001$).

In this study, we used a well-characterized anti-Rsf-1 antibody to study the expression pattern of Rsf-1 in CCC, and provided new evidence that the expression of Rsf-1 was associated with advanced clinical stages and with the status of lymph node metastasis in CCC. The findings suggest a biological role for Rsf-1 in disease aggressiveness in this type of ovarian carcinoma. Interestingly, we have reported earlier that chromosome 11q13.5 amplification and overexpression in the cases of ovarian high-grade serous carcinoma contribute to a shorter overall survival compared with the cases without amplification. A possible mechanism was thought to be related to the *de novo* paclitaxel resistance rendered by Rsf-1 overexpression (27). Although the Kaplan-Meier survival analysis did not show a statistically significant difference between the Rsf-1-positive CCC cases and the Rsf-1-negative CCC cases, long-term prognosis of the Rsf-1-positive cases seems to be slightly worse than the Rsf-1-negative cases. However, the number of Rsf-1-negative CCC cases in our series was relatively small, and we believe that analysis in a larger series on CCCs is required to conclude if Rsf-1 overexpression predicts worse overall survival in CCCs. Furthermore, our study suggests a potential use of Rsf-1 immunoreactivity as a biomarker, which may prove useful for predicting clinical outcomes in primary CCC, including higher clinical stages, and for predicting the risk of developing lymph node metastasis. To this end, several proteins including IGF2BP3 (IMP3) (11) and annexin A4 (12) have been reported as new markers associated with the treatment outcomes in CCC. Thus, a panel of different markers including Rsf-1 could be tested in future clinical trials to determine their potential for use in the management of CCC patients.

In this report, we observed that with a single exception, the immunostaining intensity score of Rsf-1 was less than 3+ in all the cases analyzed.

This finding provides an independent confirmation of our earlier observation in another, smaller set of CCC samples in which we observed that the majority of CCCs showed an immunostaining intensity score of 1+ or 2+ (14). In fact, the percentage of Rsf-1-positive and negative cases is very similar between this and the earlier report. Moreover, analysis of single nucleotide polymorphism arrays performed on affinity-purified CCC specimens did not show an increase in the DNA copy number at chromosome 11q13.5, indicating that Rsf-1 is rarely amplified in CCC (28). The above findings in CCC are in sharp contrast to those in high-grade serous carcinoma (14), and underscore the distinct molecular pathways in developing CCC and high-grade serous carcinoma [reviewed in Ref. (29)]. It is also noteworthy that endometrioid and mucinous carcinomas of the ovary express Rsf-1 much less frequently as compared with CCCs and high-grade serous carcinoma. Only 49% of endometrioid carcinomas and 48% of mucinous carcinomas were Rsf-1 positive, and the intensity scores of the positive cases were mostly 1+ and 2+.

In conclusion, using immunohistochemistry with an Rsf-1-specific antibody we showed that the presence of Rsf-1 immunoreactivity is significantly associated with advanced stage and lymph node metastasis in primary CCCs. Our findings suggest that Rsf-1 expression may contribute to disease aggressiveness in CCC, and warrant further study of the biological role of Rsf-1 in the progression of CCC.

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ORIGINAL ARTICLE

Mucosal carcinoma of the fallopian tube coexists with ovarian cancer of serous subtype only: a study of Japanese cases

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Abstract Previous studies in Western countries have revealed that mucosal carcinoma of the fallopian tube frequently coexists with pelvic (ovarian, tubal, and peritoneal) serous carcinomas, and early tubal carcinoma is now regarded as a possible origin of these tumors. However, the relationship between early tubal carcinoma and non-serous ovarian cancer, such as clear cell adenocarcinoma, has not been studied in detail. In this study, we sought to examine the coexistence of mucosal carcinoma of the fallopian tube in Japanese ovarian cancer cases. We submitted the fallopian tubes in toto for histological examination in 52 ovarian carcinoma cases and three peritoneal serous carcinoma cases. The ovarian tumors included 12 serous adenocarcinomas, 23 clear cell adenocarcinomas, nine endometrioid adenocarcinomas, three mucinous adenocarcinomas, and four mixed epithelial carcinomas. Mucosal carcinoma of the fallopian tube did not coexist with non-serous adenocarcinoma (n=40). In contrast, mucosal carcinoma of the fallopian tube was observed in six cases of ovarian serous adenocarcinoma and one case of peritoneal serous adenocarcinoma. In these cases, the p53 immunophenotypes were similar in tubal lesions and invasive ovarian or peritoneal carcinomas. Tumors were negative for p53 in four of seven cases, and one of the p53-negative serous adenocarcinomas showed low-grade morphology. We believe that some ovarian and peritoneal serous adenocarcinomas develop from early tubal carcinomas. However, it should be noted that early tubal carcinomas are not always p53-positive immunohistochemically. Finally, it is unlikely that early tubal lesions are involved in the carcinogenesis of clear cell adenocarcinoma and other non-serous adenocarcinomas.

Keywords Ovarian carcinoma · Fallopian tube · Mucosal carcinoma · p53

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Introduction

The pathogenesis of primary ovarian carcinomas has long been controversial, and it remains a topic of debate. Ovarian surface epithelium or intracortical inclusion cysts have been considered to be the origin of most ovarian carcinomas, and thus, the term "malignant surface epithelial tumor" has been used to represent ovarian carcinoma [1]. It is also known that some endometrioid adenocarcinomas

and clear cell adenocarcinomas arise from endometriotic lesions [2–4]. At this point, regarding mucinous adenocarcinomas and low-grade serous adenocarcinomas, stepwise carcinogenesis from borderline tumor to invasive carcinoma has been suggested. In contrast, high-grade serous adenocarcinoma, which often presents in an advanced stage with multiple peritoneal disseminations, is believed to arise de novo, and *TP53* mutation is known to play a key role in its progression [5, 6].

In recent years, accumulating evidence suggests that the fallopian tube epithelium is the origin of the tumors conventionally diagnosed as ovarian high-grade serous adenocarcinomas [7–15]. This novel theory has attracted much attention in the field of gynecological research. Three major findings were essential to establishing this theory. First, extensive sectioning of salpingo-oophorectomy specimens from women with BRCA mutations has shown that the fallopian tube, especially its fimbriated end, is a preferred site for early adenocarcinoma [9, 16–18]. These early forms of tubal adenocarcinoma were usually in situ lesions, and Crum et al. referred to them as "tubal intraepithelial carcinomas" (TICs) [7, 10]. Second, histological examination of the whole fallopian tube has demonstrated that TICs frequently coexisted with ovarian and peritoneal high-grade serous adenocarcinomas in the general population [8, 10]. Finally, analyses of TICs and ovarian carcinomas for p53 protein accumulation, *TP53* mutations, and chromosomal instability revealed similar genetic alterations in both tubal and ovarian lesions [8, 10, 11]. Indeed, these key findings provide evidence for designating TICs as preceding lesions of high-grade serous cancers. Nonetheless, previous reports on the association between fallopian tube lesions and ovarian and peritoneal cancers are from limited institutions in North America, and they focus primarily on serous carcinogenesis and the involvement of *TP53* gene alterations.

In the current study, we examined the coexistence of mucosal carcinoma of the fallopian tube among Japanese cases of ovarian and peritoneal cancer. The distribution of ovarian carcinoma cases according to their histological subtypes is different between Japan and Western countries. In Japan, the incidence of clear cell adenocarcinoma is much higher [19–21]. Consequently, we were able to include not only serous adenocarcinomas but also a large number of clear cell adenocarcinoma cases in this study. Our main objective was to assess whether the coexistence of mucosal carcinoma of the fallopian tube is a specific event in p53-positive high-grade serous carcinoma cases. In this report, we refrain from using the term "tubal intraepithelial carcinoma" (TIC) because it is now used widely as a term representing an early cancer that develops to form pelvic high-grade serous carcinoma, and some researchers emphasize p53 positivity as its key feature [7]. Instead, we use the term "mucosal carcinoma of the fallopian tube" to represent all histologically malignant tubal lesions that show intraepithelial growth, regardless of their immunophenotype.

Materials and methods

Case selection

We prospectively analyzed cases diagnosed as ovarian carcinoma or peritoneal serous carcinoma in four major hospitals in Tokyo, Japan (the University of Tokyo Hospital, Toranomon Hospital, Mitsui Memorial Hospital, and Teikyo University Hospital) between 2007 and 2009. The definitions of peritoneal and ovarian carcinomas were based on criteria adapted from the International Federation of Gynecology and Obstetrics (FIGO) and the World Health Organization [22, 23]. Cases with predominantly peritoneal tumor and minimal ovarian surface or tubal involvement were classified as peritoneal carcinomas. The diagnosis of ovarian carcinoma was confirmed when the bulk of the neoplasm was identified in the parenchyma of the ovary. Histological classification of the ovarian and peritoneal carcinomas was based on the most recent criteria of the World Health Organization [23]. Ovarian carcinomas diagnosed as serous adenocarcinomas were subcategorized as either low- or high-grade, according to a two-tier grading system (adapted from the MD Anderson Cancer Center grading system) [24]. Tumor staging was performed in accordance with the standards of FIGO, based on clinical information and pathological findings.

Section preparation and examination of resected specimens

For salpingo-oophorectomy specimens, we attempted to submit the fallopian tube, including the fimbria, in toto. The fallopian tubes were sectioned serially, approximately every 3 mm. The histological diagnosis of mucosal (in situ) carcinoma of the fallopian tube was based solely on morphology. Mucosal carcinoma was characterized by replacement of the normal tubal epithelium by malignant glandular epithelial cells with pleomorphic nuclei [23]. Stratification, loss of epithelial polarity, and lack of stromal invasion are other features of mucosal carcinoma of the fallopian tube. When peritoneal dissemination was sampled and submitted separately to the Department of Pathology, we evaluated its representative sections. The presence or absence of peritoneal dissemination was recorded in all the cases.

Completeness of tubal sectioning

We graded the completeness of the in toto tubal sectioning using a four-tier system, as described in Table 1. Of the 71

Table 1 Grading of completeness of in toto sectioning of the fallopian tubes and case distribution

	Submitted adnexa	Tumor involvement	In toto sectioning	Cases (n=71)
A (perfect)	Bilateral	Ovary: unilateral/bilateral Peritoneal dissemination: +/−	Completely performed bilaterally	40
B (satisfactory)	Unilateral	Ovary: clinically unilateral Peritoneal dissemination: −	Completely performed unilaterally (affected side)	8
	Bilateral	Ovary: histologically unilateral Peritoneal dissemination: −	Completely performed unilaterally (affected side)	1
C (adequate)	Bilateral/ unilateral	Ovary: histologically or clinically unilateral Peritoneal dissemination: +	Completely performed unilaterally (affected side)	3
		Ovary: histologically or clinically bilateral Peritoneal dissemination: +/−	Completely performed unilaterally (ipsilateral to the dominant ovarian mass)	3
D (inadequate)	Bilateral/ unilateral	Ovary: unilateral/bilateral Peritoneal dissemination: +/−	Incompletely performed in the tube ipsilateral to the dominant ovarian mass	16

MCFT mucosal carcinoma of the fallopian tube

ovarian and peritoneal cancer cases retrieved, we were able to perform appropriate sectioning in 55 cases (Grades A to C). As for peritoneal cancer cases, sectioning was considered appropriate only when bilateral fallopian tubes were perfectly sectioned. Three peritoneal serous adenocarcinoma cases were classified in the Grade A category. Reasons for incomplete sectioning (Grade D) included an unidentifiable fallopian tube due to replacement by tumor and inadequate sections made in the initial settings. Further clinicopathological analyses including a survey for mucosal carcinoma of the fallopian tube were performed on cases classified as Grades A to C.

Clinical survey of patients with serous adenocarcinoma

We examined the medical records of 15 serous adenocarcinoma (12 ovarian and three peritoneal) patients and their demographics: data including age, tumor site, preoperative diagnosis, survival status, and follow-up period were obtained. None of the patients underwent preoperative chemotherapy or radiotherapy. The follow-up period was calculated from the date of surgery to the date of death or last clinical evaluation. The mean follow-up interval was 11.9 months (range, 1-33). Medical records of non-serous adenocarcinoma patients were not investigated in this study.

Immunohistochemistry

Tissue samples were fixed in formalin and embedded in paraffin. For the serous adenocarcinoma cases, sections (4 μm) were cut from paraffin-embedded blocks containing ovarian or peritoneal tumor tissue and mucosal carcinoma of the fallopian tube, when present. Immunohistochemistry was performed with the following antibodies: mouse monoclonal anti-p53 antibody (1:100, Clone DO-7, Novocastra Laboratories, Newcastle Upon Tyne, UK), mouse monoclonal anti-Ki67 antibody (1:100, Clone MIB-1, Dako, Glostrup, Denmark), and mouse monoclonal anti-Wilms' tumor 1 (WT-1) protein (1:25, Clone 6F-H2, Dako). Immunohistochemical staining was performed according to standard techniques using a Ventana Benchmark® XT autostainer (Ventana Medical Systems Inc, Tucson, AZ, USA). Appropriate controls were included. p53 immunoreactivity was classified as completely negative (CN) when no cells showed any positive nuclear immunoreactivity, negative (N) when nuclear immunoreactivity was observed in <10% of cells, and positive (P) if ≥10% of cells showed strong nuclear immunoreactivity [25]. The percentage of cells with positive Ki-67 nuclear staining was interpreted as the proliferation index (MIB-1 index) for each lesion. MIB-1 index was quantified by counting at least 200 cells in the most immunoreactive area in each sample. As for minute lesions that consisted of less than 200 cells, all the cells were subjected to analysis. WT-1 immunoreactivity was assessed based on nuclear staining, and its intensity was classified subjectively as weak or strong. WT-1 immunoreactivity was scored as follows: 0 (totally negative or <10% of cells strongly/weakly positive), 1+ (≥10% of cells weakly positive and/or 10–50% of cells strongly positive), or 2+ (>50% of cells strongly positive). Additionally, the immunoreactivity of benign epithelium on the same sections, but away from mucosal carcinoma of the fallopian tube, was assessed with all three antibodies.

Examination of fallopian tubes for p53 signatures

To clarify the incidence of benign epithelium showing linear p53 immunopositivity (p53 signature) in the background tubal mucosa of serous adenocarcinoma cases and non-serous adenocarcinoma cases, we performed immunohistochemistry

for p53 in all the fallopian tube tissues sectioned in all of the serous adenocarcinoma cases (n=15) and in 15 cases of clear cell adenocarcinoma chosen as a control group. We made the diagnosis of p53 signature when greater than 75% of benign-looking tubal epithelial cells showed strong immunoreactivity for p53 in a linear manner, exceeding 12 cells in length, in accordance with a previous report [26].

Statistical analysis

Statistical analysis was performed using the χ^2 test. Statistical analyses were performed with StatView 5.0 software (SAS Institute, Cary, NC, USA), and $P<0.05$ was considered statistically significant.

Results

Ovarian and peritoneal carcinoma case distribution and presence of mucosal carcinoma of the fallopian tube

The distribution of the ovarian and peritoneal carcinoma cases and frequency of coexisting mucosal carcinoma of the fallopian tube are summarized in Table 2. Of the 55 cases properly examined for tubal lesions, three were peritoneal high-grade serous carcinoma cases and 52 were ovarian carcinoma cases. Histologically, our series consisted of 15 serous adenocarcinomas (14 high-grade serous adenocarcinomas and one low-grade serous adenocarcinoma), 23 clear cell adenocarcinomas, nine endometrioid adenocarcinomas, two mucinous adenocarcinomas, one mucinous borderline tumor with intraepithelial carcinoma, four mixed epithelial carcinomas, and one undifferentiated carcinoma. Perfect sectioning of the fallopian tubes (Grade A) was performed in 13/15 serous adenocarcinoma cases, and in 27/40 non-serous adenocarcinoma cases (P=0.155). Mucosal carcinoma of the fallopian tube was present only in the serous adenocarcinoma cases (7/15) and not in the non-serous adenocarcinoma cases (0/40; P=0.0001). We observed coexisting mucosal carcinoma of the fallopian tube in five ovarian high-grade serous adenocarcinoma cases, one ovarian low-grade serous adenocarcinoma case, and one peritoneal serous adenocarcinoma case. Perfect sectioning of the fallopian tubes (Grade A) was performed in all seven serous adenocarcinoma cases that had coexisting tubal lesions. Of the eight serous adenocarcinomas without coexisting tubal carcinoma, six were in the Grade A (perfect) sectioning category and two were in the Grade C (adequate) sectioning category. Statistically, grade of sectioning was not significantly different between serous adenocarcinoma cases with coexisting tubal lesions and those without (P=0.155).

Clinicopathological features of serous adenocarcinoma patients

Since early tubal carcinomas were associated only with ovarian/peritoneal serous adenocarcinomas, we specifically examined medical records of serous adenocarcinoma cases. The clinicopathological features including the immunophenotypes of 15 serous adenocarcinomas are summarized in Table 3. Cases 1–7 are cases with mucosal carcinomas of the fallopian tube, and cases 8–15 are those without. Histologically, all of the serous carcinomas except for case 1 were high-grade serous adenocarcinomas.

In case 1, the ovarian tumor showed features of low-grade serous adenocarcinoma, such as mild nuclear atypia, low mitotic activity (7 per 10 HPFs), coexistence of serous borderline (atypical proliferative) tumor component, and p53-negative (N) immunophenotype (Fig. 1). Its predominant architectural pattern was glandular and papillary pattern. A solid component was present, but focal.

In all ovarian serous adenocarcinoma cases with mucosal carcinomas of the fallopian tube, coexisting tubal lesions were found in the fimbriae ipsilateral to the dominant ovarian tumor. Mucosal carcinomas of the fallopian tube were multifocal in cases 1 and 2. In case 1, another mucosal carcinoma was found in non-fimbriated tubal mucosa ipsilateral to the dominant ovarian tumor. In case 2, ovarian carcinoma showed bilateral involvement, and minute mucosal carcinoma of the fallopian tube was also found in the fimbria contralateral to the dominant ovarian mass. With regard to patient age, tumor stage, and survival, we found no obvious difference between cases with and without mucosal carcinoma of the fallopian tube.

Histologically, in cases 2–4, the mucosal carcinomas of the fallopian tube in the ipsilateral side of the dominant

Table 2 Coexistence of mucosal carcinoma of the fallopian tube in ovarian and peritoneal cancer cases

Histology	Serous (n=15)	Non-serous (n=40)			
		Clear cell	Endometrioid	Mucinous	Others
Number of cases	15	23	9	3	5
Peritoneal dissemination (+)	13	7	0	0	1
Mucosal carcinoma of the fallopian tube (+)	7	0	0	0	0

Table 3 Clinicopathological and immunohistochemical features of serous adenocarcinoma cases

Case	Diagnosis	Age	Histological grade	Stage	Survival	PD	Sig	MCFT	p53 status	WT-1 status	MIB-1 index
1	OC	46	Low	III	NED (20 months)	+	−	+	N/N/N[a]	1+/2+/2+[a]	18/9/1[a]
2	OC	38	High	III	DOD (7 months)	+	−	+	P/P/N[a]	−/−/2+[a]	76/72/2[a]
3	OC	48	High	III	NED (2 months)	+	−	+	N/N/N[a]	2+/2+/2+[a]	71/54/1[a]
4	OC	46	High	III	AWD (15 months)	+	−	+	CN/CN/N[a]	2+/2+/1+[a]	56/55/1[a]
5	OC	47	High	III	NED (4 months)	+	−	+	CN/CN/N[a]	2+/2+/1+[a]	76/40/1[a]
6	OC	57	High	III	AWD (3 months)	+	+	+	P/P/N[a]	1+/2+/2+[a]	34/14/3[a]
7	PC	66	High	III	AWD (1 months)	+	+	+	P/P/N[a]	1+/2+/2+[a]	75/29/0[a]
8	OC	55	High	III	NED (19 months)	−	−	−	P[b]	1+[b]	46[b]
9	OC	59	High	I	NED (17 months)	−	−	−	P[b]	2+[b]	51[b]
10	OC	49	High	III	NED (15 months)	+	−	−	P[b]	2+[b]	44[b]
11	OC	35	High	IV	AWD (8 months)	+	−	−	P[b]	2+[b]	11[b]
12	OC	58	High	III	NED (9 months)	+	−	−	P[b]	2+[b]	42[b]
13	OC	38	High	III	NED (7 months)	+	+	−	N[b]	1+[b]	49[b]
14	PC	49	High	III	NED (18 months)	+	+	−	CN[b,c]	2+[b]	47[b]
15	PC	66	High	IV	AWD (33 months)	+	−	−	CN[b]	2+[b]	78[b]

PD peritoneal dissemination, *Sig* p53 signature, *MCFT* mucosal carcinoma of the fallopian tube, *WT-1* Wilms' tumor 1, *IT* invasive (ovarian or peritoneal) tumor, *BTE* benign tubal epithelium, *OC* ovarian carcinoma, *PC* peritoneal carcinoma, *NED* no evidence of disease, *AWD* alive with disease, *DOD* died of disease, *CN* completely negative, *N* negative, *P* positive

[a] IT/MCFT/BTE

[b] IT

[c] Completely negative except for one tubal lesion showing diffuse p53 positivity

ovarian tumor were in situ lesions by themselves, and no invasive carcinoma was found in continuity with them. In cases 1, 5, and 7, invasive tubal carcinoma was found in continuity or adjacent to the in situ lesions. Histology of the mucosal carcinoma of the fallopian tube was similar to that of the coexisting dominant invasive carcinoma mass in the ovary or peritoneum.

In case 1, the tubal lesion also had low-grade morphological features (Fig. 2). Uniform tumor cells with mild nuclear atypia replaced the tubal mucosa, and they displayed a prominent papillary growth pattern. Its histology was quite similar to that of a typical ovarian serous borderline tumor. Mucosal carcinomas of the fallopian tube in cases 2–7 were composed of tumor cells with marked nuclear atypia (Fig. 3). The presence of prominent nucleoli and frequent mitotic activity were also observed. In cases 3-7, mild to moderate inflammatory cell infiltration was observed in the stroma beneath the mucosal carcinomas of the fallopian tube. In some of these cases, there were histologically obvious cancer implantations in nearby tubal mucosa that were surrounded by desmoplastic stroma.

Immunohistochemical features of serous adenocarcinoma cases

Immunohistochemically, mucosal carcinomas of the fallopian tube that coexisted with ovarian/peritoneal serous adenocarcinomas showed higher MIB-1 index, compared with the benign fallopian tube epithelium (Figs. 2b and 3f). In all seven cases with coexisting mucosal carcinoma of the fallopian tube, both tubal lesions and invasive ovarian/peritoneal carcinomas showed similar immunophenotypes for p53 and WT-1. In case 1, the immunophenotype was only assessable in one of two coexisting mucosal carcinomas of the fallopian tube because the other tubal lesion disappeared in sections for immunohistochemistry. In case 2, two mucosal carcinomas of the fallopian tube showed similar immunophenotypes. Surprisingly, diffuse nuclear staining for p53 protein was observed only in three cases of ovarian/peritoneal carcinomas with coexisting tubal carcinomas (cases 2, 6, and 7; Fig. 3d). In the rest four cases, both invasive ovarian cancer and coexisting tubal carcinoma showed negative (either CN, completely negative; or N, negative) immunoreactivity for p53. Among them, cases 4 and 5 revealed completely negative (CN) immunophenotype for p53, and cases 1 and 3 showed negative (N) immunoreactivity (i.e., only scattered positive cells) for p53 (Figs. 1d and 2a).

Among eight ovarian and peritoneal serous carcinomas that had no coexisting mucosal carcinoma of the fallopian tube, five cases showed positive immunoreactivity for p53, one case revealed p53-completely negative (CN) immunophenotype, and one case was p53-negative (N). However, one remaining case (case 14) revealed an exceptional p53 immunophenotype. Case 14 was diagnosed as peritoneal high-grade serous adenocarcinoma. Along with many

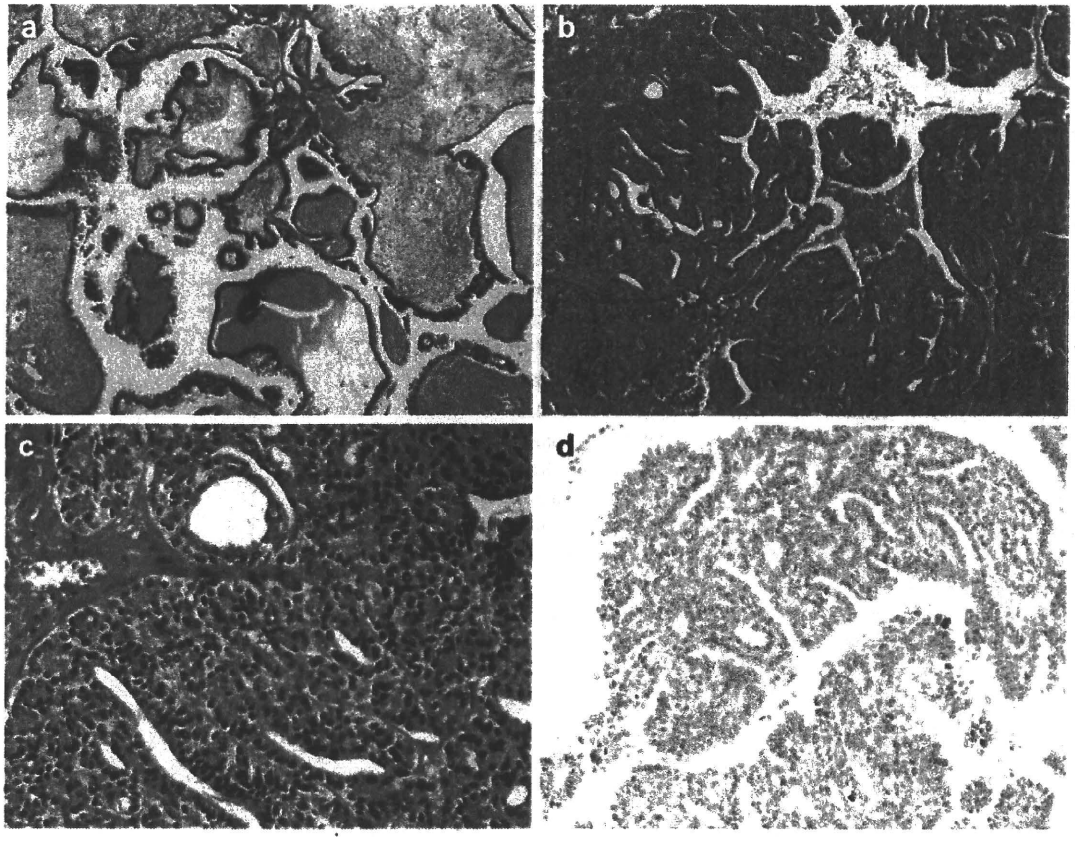

Fig. 1 Histology of ovarian tumor in case 1. **a** Serous borderline tumor component that coexisted with low-grade serous adenocarcinoma. **b** Majority of the ovarian mass was composed of low-grade serous adenocarcinoma showing papillary and glandular patterns of growth. **c** High-power view of the low-grade serous adenocarcinoma. Tumor cells are monotonous and their atypia is mild. **d** Low-grade serous adenocarcinoma showing only scattered immunoreactivity for p53

tumor foci in the omentum (Fig. 4a), we observed superficial involvement of bilateral ovaries (Fig. 4b) and a round discrete tumor nest in the submucosal stroma of the left tube (Fig. 4c). Although these tumor foci (omental, ovarian, and tubal) revealed similar morphological features, the p53 immunophenotype was significantly different between the omental/ovarian carcinoma and the left tubal carcinoma. After immunostaining all the slides that contained carcinoma, we found that all omental and ovarian carcinoma foci were completely negative (CN) for p53 (Fig. 4a, b) and that the left tubal carcinoma focus was diffusely positive for p53 (Fig. 4c). We then made serial sections of the left tubal lesion to examine whether mucosal carcinoma was in adjacent tubal mucosa. Deeper sections revealed tumor exposure in the tubal mucosal (Fig. 4d), but no in situ carcinoma component was identified.

p53 signature in the background tubal mucosa of serous and clear cell adenocarcinoma cases

The p53 signature was present in four of 15 cases of serous adenocarcinoma (Table 3). Of the cases with p53 signatures, two (cases 6 and 7) had coexisting mucosal carcinoma of the fallopian tube. Among the control group of clear cell adenocarcinoma cases, p53 signatures were found in seven of 15 cases (Fig. 5). The p53 signatures were often multifocal and, in some cases, bilateral. Of the 17 p53 signatures (five in serous adenocarcinoma cases and 12 in clear cell adenocarcinoma cases) detected in our series, 14 were located in the non-fimbriated tubal mucosa, and only three were in the fimbriated ends. The MIB-1 indices of the p53 signatures were very low, mostly below 5%, and no increase was observed in comparison with the index of the adjacent tubal mucosa.

Discussion

A newly introduced theory that designates the origin of ovarian cancer to the tubal epithelium has attracted significant attention. The theory is expected to explain, at least to some extent, the ovarian carcinogenesis that had for the most part been a mystery. However, it should be noted that only a few studies have assessed the association

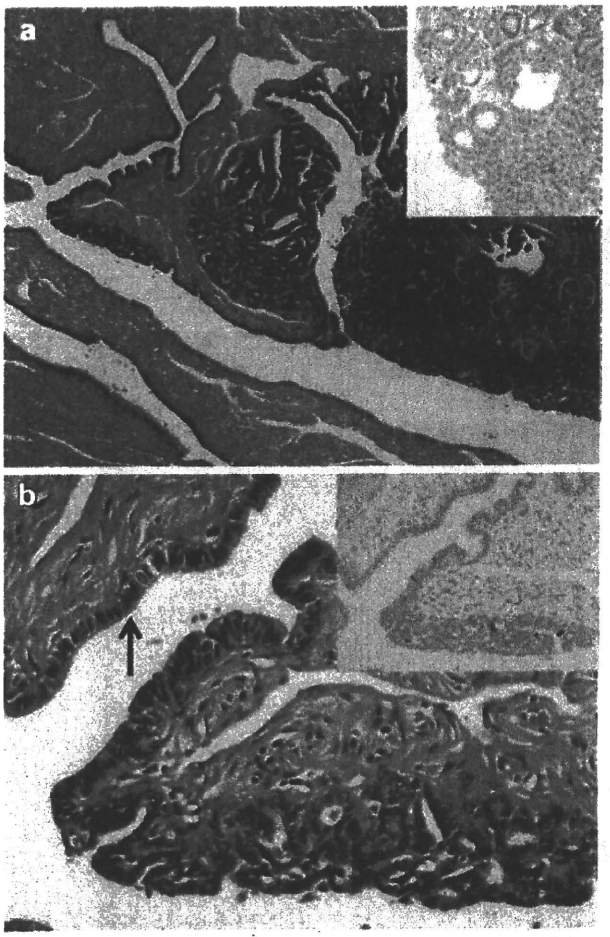

Fig. 2 Mucosal carcinoma of the fallopian tube detected in case 1. **a** Low-power view of the tubal low-grade serous adenocarcinoma. Predominant component of the tumor was noninvasive (in situ). However, some foci of microinvasion were observed. Tubal carcinoma was negative for p53 (*inset*). **b** High-power view of the tubal low-grade serous carcinoma and benign background tubal mucosa (*arrow*). Tumor cells with only mild atypia grew in complexed glandular structures and papillary structures. Immunostaining for Ki-67 is shown in the *inset*. Although the tubal carcinoma showed higher MIB-1 index in comparison with the benign tubal epithelium, its MIB-1 index was no more than 5%

between mucosal carcinomas of the fallopian tube and ovarian carcinomas in general populations to date [8, 10, 11, 15]. In these reports, the authors focused primarily on high-grade serous carcinogenesis and p53 alterations involved in the process. Other histological subtypes of ovarian cancer have not been studied in detail for coexisting tubal lesions. In fact, none of the past series included ovarian clear cell adenocarcinomas, which sometimes resemble serous adenocarcinoma histologically and pose difficult problems to surgical pathologists. In this study, we examined Japanese ovarian and peritoneal cancer cases, which included a large number of ovarian non-serous adenocarcinomas. The high prevalence of clear cell adenocarcinomas in our series should be attributed to the Japanese patient background. By comparing four major histological subtypes of ovarian carcinoma (serous, clear cell, endometrioid, and mucinous), we demonstrated that the coexistence of mucosal carcinoma of the fallopian tube was specific to cases of serous adenocarcinoma. The results of our study suggest that tubal lesion is unlikely to be associated with the development of ovarian clear cell adenocarcinomas and other non-serous adenocarcinomas. Thus, with regard to the origin of non-serous ovarian cancer, attention should rather be paid to the ovarian surface epithelium, inclusion cysts, and endometriotic lesions.

In our study, coexisting mucosal carcinoma of the fallopian tube was found in seven of 15 serous adenocarcinoma cases. The incidence of coexisting mucosal carcinoma and frequency of fimbrial involvement were similar to the values reported previously [8, 10, 15]. However, the p53 immunophenotype of mucosal carcinomas of the fallopian tube in our series was different from those in previous reports. In contrast to the previous series, which showed that nearly all mucosal carcinomas of the fallopian tube were immunohistochemically p53-positive [8, 10], more than half of the tubal lesions in our series were p53-negative. In this report, we hope to emphasize the existence of p53-negative mucosal carcinomas of the fallopian tube. Considering that more than 30% of high-grade serous adenocarcinomas are p53-negative immunohistochemically [26], it is reasonable that we encounter occasional p53-negative early tubal carcinomas that coexist with p53-negative high-grade ovarian/peritoneal serous adenocarcinomas. In general, a cautious approach is necessary in the evaluation of p53 immunoreactivity, especially when assessing the involvement of *TP53* mutations. It is well known that diffuse nuclear p53 immunoreactivity suggests the presence of such mutations. Importantly, studies have revealed that protein-truncating *TP53* mutations can lead to completely negative immunoreactivity [27]. On the other hand, a few scattered p53-positive cells by immunohistochemistry suggest that the lesion harbors wild-type *TP53*. In our series, most of the high-grade serous adenocarcinoma cases, including their coexisting tubal lesions, scored either as P (positive) or CN (completely negative) in immunoreactivity for p53. Therefore, it is highly likely that these tumors harbor *TP53* mutations, and we believe *TP53* alterations play a significant role in the tumorigenesis of many tubal mucosal carcinomas.

Another striking finding in our study was the presence of tubal mucosal carcinoma coexisting with ovarian low-grade serous adenocarcinoma. Currently, not much is known about tubal low-grade serous adenocarcinoma, and there has been no report on the coexistence of tubal mucosal carcinoma in an ovarian low-grade serous adenocarcinoma

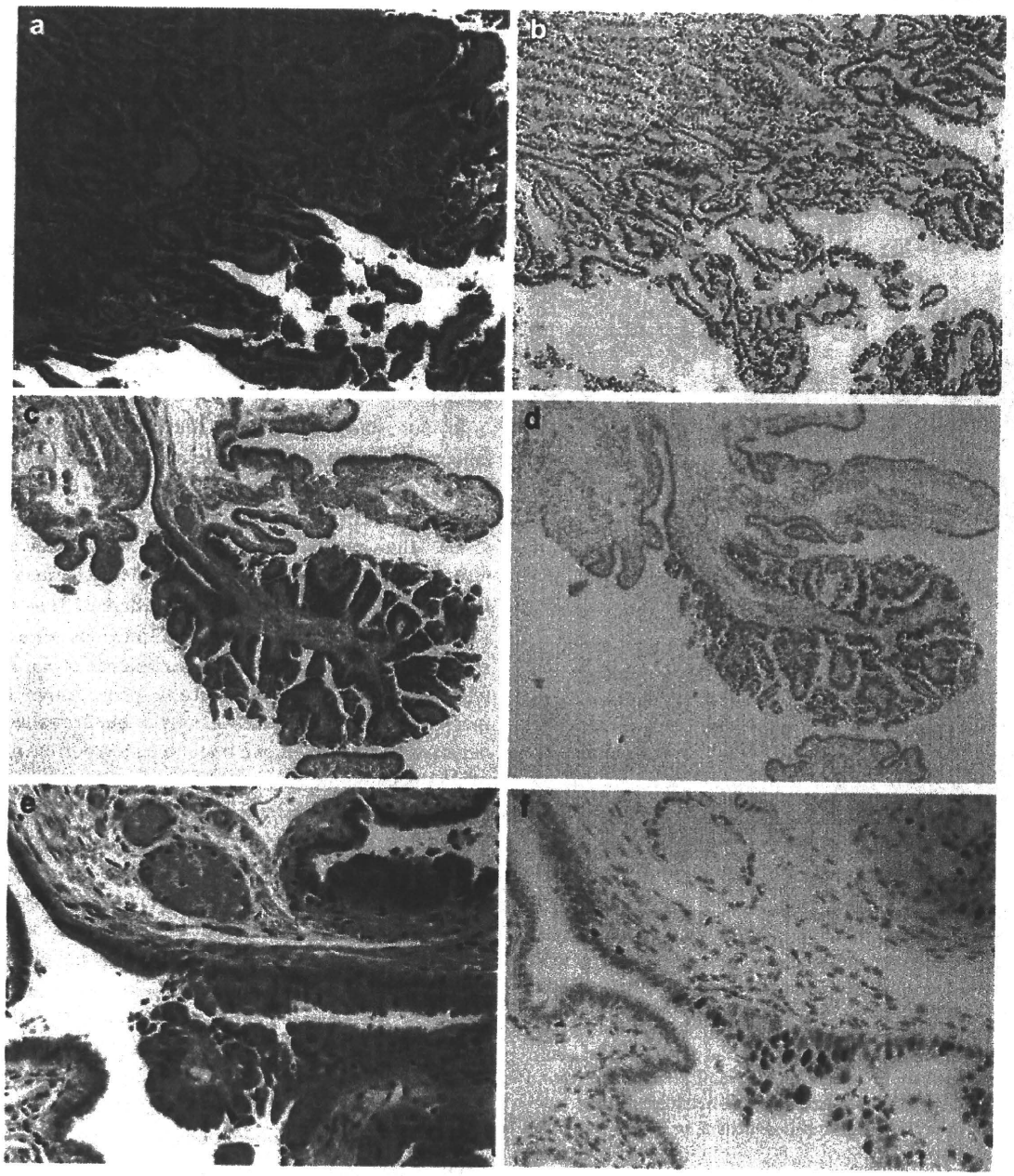

Fig. 3 Histology of ovarian tumor and coexisting mucosal carcinoma of the fallopian tube in case 2. **a** Ovarian carcinoma was high-grade serous adenocarcinoma showing papillary pattern of growth. **b** Ovarian high-grade serous adenocarcinoma showing diffuse immunoreactivity for p53. **c** Mucosal carcinoma of the fallopian tube detected in the fimbriae. **d** Mucosal carcinoma of the fallopian tube was p53-positive. **e** High-power view of the mucosal carcinoma of the fallopian tube. Tumor cells with moderate to severe atypia showing intraepithelial growth. **f** MIB-1 index of the mucosal carcinoma of the fallopian tube was elevated markedly (>50%), compared to adjacent benign mucosa

case. In our case, the tubal lesion showed prominent papillary growth and was large enough to be readily recognized upon microscopic examination. However, cytological atypia of this low-grade tubal carcinoma was very mild, and only a slight increase in MIB-1 index was seen. In addition, p53 immunohistochemistry revealed a wild-type *TP53* phenotype. Based on our experience, we believe that such low-grade tubal lesions can be diagnosed only by means of thorough histological examination, as immunohistochemistry may not always be helpful. Initially, surgical pathologists should be made aware of the occurrence of tubal low-grade serous adenocarcinoma. Accumulation of information about low-grade tubal carcinomas will provide more insights into the pathogenesis of early tubal cancers,

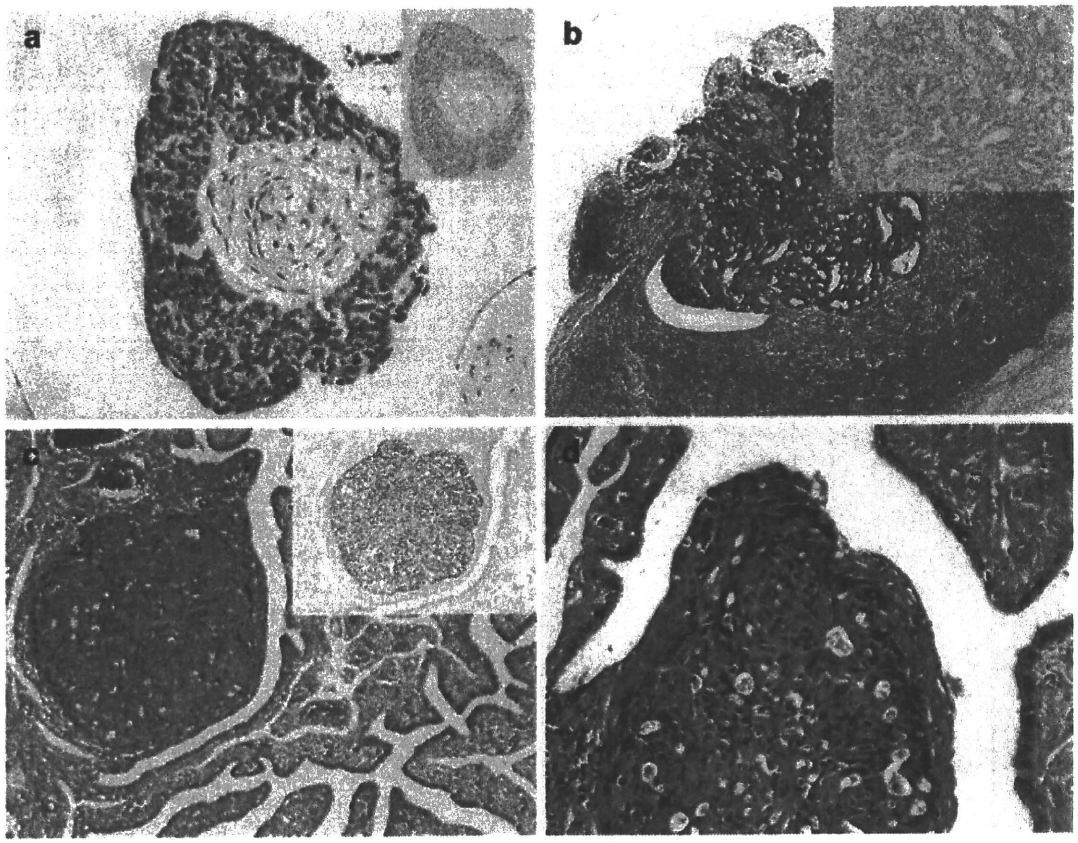

Fig. 4 Histological features of pelvic serous adenocarcinomas of case 14. **a** Nests of serous adenocarcinoma in the omentum, and p53 immunostaining (*inset*). Adenocarcinoma in the omentum is completely negative for p53. **b** Serous adenocarcinoma involving the ovarian surface and p53 immunostaining (*inset*). Adenocarcinoma in the ovary is also completely negative for p53. **c** A well-circumscribed nodule of serous adenocarcinoma detected in the submucosa of the left fallopian tube, and p53 staining (*inset*). The tubal lesion is diffusely positive for p53. **d** A deeper section of the left tubal carcinoma revealed tumor exposure in the tubal mucosa. However, no intraepithelial carcinoma was detected in the adjacent tubal mucosa

including their potential association with ovarian low-grade serous adenocarcinomas.

Regarding p53 alterations in the fallopian tube, we also evaluated the existence of p53 signatures in the background tubal mucosa. The p53 signature has been recognized as a lesion that will evolve to p53-positive mucosal carcinoma of the fallopian tube [14]. Studies have emphasized its importance as an initial change that occurs in pelvic serous carcinogenesis [11, 28]. Interestingly, the p53 signature has been reported to be equally prevalent in benign tubes of BRCA+ and control women [11]. In this series, the prevalence of p53 signatures was not significantly different between serous adenocarcinoma cases and clear cell adenocarcinoma cases. Based on this result, we assume that the presence of the p53 signature itself does not constitute a risk for developing p53-positive pelvic serous carcinoma. Rather, events that take place between the p53 signature and p53-positive mucosal carcinoma of the fallopian tube should be regarded as key steps in the carcinogenesis of p53-positive pelvic serous adenocarcinomas.

Theoretically, there are three ways of interpreting mucosal carcinoma of the fallopian tube: most importantly, as an "early carcinoma (preceding lesion) that progresses to form pelvic serous carcinoma," as a "disseminated serous carcinoma showing intraepithelial spread," or as a part of "multifocal (multiclonal) neoplastic lesions in the pelvis." Each theory has its strong points and weak points. Recent studies have provided evidence in support of the "preceding lesion" theory [7–11, 15]. The frequent presence of early tubal lesions in prophylactically removed specimens from BRCA carriers is an important rationale for designating mucosal carcinoma of the fallopian tube as a "preceding lesion," not just a "coexistent lesion." However, BRCA carriers comprise only about 10% of ovarian cancer patients [29–31], and incidence of mucosal carcinoma of the fallopian tube in patients without BRCA mutations remains unclear. In addition, the precise events that take place between the evolution of initial early tubal carcinoma and the development of mass-forming ovarian or peritoneal serous carcinoma have yet to be clarified. Thus, although

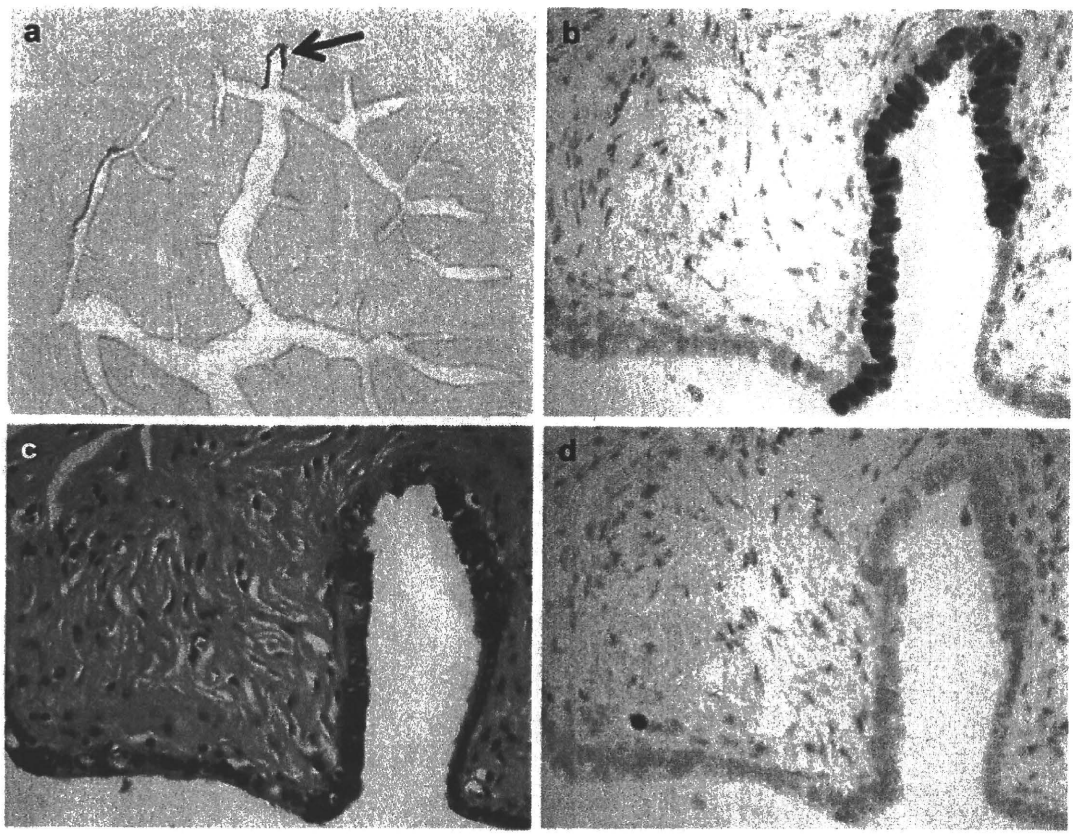

Fig. 5 p53 signatures in the background tubal epithelium of an ovarian clear cell adenocarcinoma case. **a** Immunostaining for p53. Multifocal p53 signatures were found in non-fimbriated tubal mucosa. **b** High-power view of a p53 signature (*arrow* in **a**), (**c**) its hematoxylin and eosin staining, and (**d**) its Ki-67 immunostaining. MIB-1 index of the p53 signature was 0%

we believe, based on the results of our study, that mucosal carcinoma of the fallopian tube is a candidate preceding lesion of pelvic serous adenocarcinoma, additional evidence is necessary to strengthen this hypothesis.

The possibility of some tubal mucosal carcinomas being cancer disseminations from remote tumors should also be discussed. In our series, seven of 13 serous adenocarcinomas with peritoneal disseminations had coexisting mucosal carcinomas of the fallopian tube. On the other hand, mucosal carcinoma of the fallopian tube was not identified in any of the eight non-serous adenocarcinomas with peritoneal disseminations. This result suggests that, in general, ovarian cancer dissemination rarely shows tubal intraepithelial spread. Consequently, it is rather unlikely that a significant number of mucosal carcinomas of the fallopian tube are disseminated carcinoma showing intraepithelial spread. However, in some of our cases, we experienced certain difficulties in making a strict histological distinction between primary intraepithelial neoplasm of the fallopian tube and cancer implantation involving the tubal mucosa. The distinction was especially problematic when the lesions were accompanied by inflammatory stroma and adjacent invasive carcinoma. Ancillary tests, such as immunohistochemistry and genetic studies, are not helpful in making such a distinction.

Finally, mucosal carcinoma of the fallopian tube might be viewed as a part of multifocal (multiclonal) pelvic tumors. Although previous molecular studies on mucosal carcinomas of the fallopian tube have indicated monoclonal origin of the tubal and ovarian tumors in most cases [8, 10, 11], the numbers of cases studied were small, and there were some exceptions. In a series by Salvador et al. [8], fluorescence in situ hybridization (FISH) analysis revealed the possible multiclonal origin of carcinomas involving the ovary and the fallopian tube in one of the cases. In another series, by Kindelberger et al. [10], a case of ovarian serous adenocarcinoma accompanied by two multiclonal tubal carcinomas was included. We have also seen a case of bilateral early tubal carcinomas with an intraepithelial component in the past, which suggested multifocal carcinogenesis in the fallopian tubes [32]. In addition, there has been a report on multiclonal origin for some peritoneal serous carcinomas in patients with BRCA1 mutation [30]. In the present study, the possibility of multiclonal serous

carcinogenesis was seriously considered in case 14, in which we evaluated numerous serous adenocarcinoma foci in the pelvis and found only one minute nodule in the left tube that was p53-positive. We did not identify a definite TIC component adjacent to the left tubal lesion. However, it is likely that the left tubal lesion is a primary p53-positive tubal serous adenocarcinoma that is not associated with p53-negative peritoneal and ovarian serous adenocarcinoma within the same patient. Another case in which we suspected multifocal carcinogenesis was case 1. In case 1, the transition between the serous borderline tumor component and the low-grade serous adenocarcinoma component was observed in the ovary. Given that a serous borderline tumor is a well-known precursor of ovarian low-grade serous adenocarcinoma, the histological findings of the ovarian tumor in case 1 are indicative of primary ovarian origin, and there is a significant chance that the coexisting early tubal carcinoma has arisen independently. Further molecular investigations including mutation analysis of genes involved in carcinogenesis of high-grade/low-grade serous adenocarcinomas (e.g., *TP53*, *BRAF*, and *KRAS*), LOH (loss of heterozygosity), and DNA copy number analysis are needed to assess the clonality of these multifocal pelvic carcinomas.

In conclusion, we demonstrated that mucosal carcinomas of the fallopian tube often coexist with the serous subtype of ovarian cancer, but not with clear cell adenocarcinoma and other non-serous adenocarcinomas of the ovary, in the Japanese population. In comparison with previous studies, tubal lesions in our series showed p53 protein overexpression less frequently. We also encountered a rare case of ovarian low-grade serous adenocarcinoma that coexisted with low-grade tubal carcinoma. Mucosal carcinoma of the fallopian tube is certainly a candidate origin (i.e., early manifestation) of ovarian and peritoneal serous carcinomas. However, to truly demonstrate its role as a preceding lesion, there are obstacles to overcome. Our observations indicate the possible heterogeneity of the lesions currently regarded as TICs or mucosal carcinomas of the fallopian tube on a histological basis. In other words, a few of them may represent a part of multiclonal pelvic serous tumors, and a very few others may be cancer dissemination showing tubal intraepithelial spread. We hope that larger studies from around the world will provide fresh and more comprehensive views on this issue. Finally, the fact that about half of the ovarian serous adenocarcinomas do not have coexisting tubal lesions should not be disregarded. At this point, the conventional de novo pathway that considers the origin of serous adenocarcinoma to be in the ovarian surface epithelium cannot be completely eliminated from the list.

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Conflict of interest None.

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ORIGINAL ARTICLE

The cell polarity regulator hScrib controls ERK activation through a KIM site-dependent interaction

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The cell polarity regulator, human Scribble (hScrib), is a potential tumour suppressor whose loss is a frequent event in late-stage cancer development. Little is yet known about the mode of action of hScrib, although recent reports suggest its role in the regulation of cell signalling. In this study we show that hScrib is a direct regulator of extracellular signal-regulated kinase (ERK). In human keratinocytes, loss of hScrib results in elevated phospho-ERK levels and concomitant increased nuclear translocation of phospho-ERK. We also show that hScrib interacts with ERK through two well-conserved kinase interaction motif (KIM) docking sites, both of which are also required for ERK-induced phosphorylation of hScrib on two distinct residues. Although wild-type hScrib can down-regulate activation of ERK and oncogenic Ras co-transforming activity, an hScrib mutant that lacks the carboxy terminal KIM docking site has no such effects. These results provide a clear mechanistic explanation of how hScrib can regulate ERK signalling and begin to explain how loss of hScrib during cancer development can contribute to disease progression.
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Keywords: hScrib; phosphorylation; ERK; protein kinase A

Introduction

The mitogen-activated protein kinase (MAPK) pathways that activate extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK) and p38 kinases have important roles in modifying the morphogenetic and motile responses of cells. Among these pathways, the Ras/Raf/MEK/ERK signal transduction cascade is a key mechanism for regulating cell fate in response to growth, proliferation, differentiation and survival signals (Fang and Richardson, 2005; Kolch, 2005; Torii *et al.*, 2006; Yoon and Seger, 2006). Activation of the cascade ultimately results in the activation of ERK and its dissociation from the MEK–ERK complex, which then stimulates gene expression, cytoskeletal rearrangements and cell metabolism, coordinating the cell's responses to a variety of extracellular signals (Schaeffer and Weber, 1999; Fincham *et al.*, 2000). Aberrations in ERK1/2 signalling are also known to be involved in a wide range of pathologies, including many cancers, diabetes, viral infections and cardiovascular disease. This pathway is hyperactivated in many tumours, with activating mutations of Ras occurring in approximately 15–30% of all human cancers (Malumbres and Barbacid, 2003; Garnett *et al.*, 2005).

Recent studies have shown that proteins involved in the regulation of cell polarity can also affect cell signalling cascades. Two of the most well-characterized of these proteins are human discs large (hDlg) and human Scribble (hScrib). In *Drosophila*, these proteins cooperate to regulate pathways of cell polarity and cell proliferation control (Bilder *et al.*, 2000; Bilder, 2004; Zeitler *et al.*, 2004). In humans, the function of these proteins is less clear. However, both are targets for several human tumour viruses and the expression of both hDlg and hScrib is frequently lost during the later stages of malignant progression, suggesting that they possess potential tumour suppressor functions in human cells (Kiyono *et al.*, 1997; Gardiol *et al.*, 1999, 2006; Nakagawa and Huibregtse, 2000; Nakagawa *et al.*, 2004; Navarro *et al.*, 2005; Nagasaka *et al.*, 2006). In the case of hDlg, multiple phosphorylation events by p38γ and JNK have been shown to regulate its localization (Sabio *et al.*, 2005; Massimi *et al.*, 2006) and recent studies have also shown that the entire hScrib cell polarity complex, comprising hDlg, hScrib and Hugl1 (human lethal giant larvae), is dynamically regulated after activation of the MAPK signalling cascade (Massimi *et al.*, 2008). A more direct effect of hScrib on the regulation of this cascade has also been shown. In one study, hScrib was shown to be able to inhibit signalling downstream of Ras and Raf, but upstream of ERK (Dow *et al.*, 2008), with loss of hScrib enhancing Ras-induced cell invasion. In a separate study, hScrib was also shown to be involved in regulating oncogene-induced apoptosis in a JNK-dependent manner, with loss of Scribble cooperating with c-myc in a mouse model of mammary carcinogenesis (Zhan *et al.*, 2008).

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All of the above data indicate that Scribble can act by modulating MAPK signalling and show a clear role for Scribble as a suppressor of tumour invasion. However, in the context of the Ras/Raf/MEK/ERK signalling module, there is no information as to how this is inhibited by Scribble. In this study, we now show that hScrib downregulates ERK activation and inhibits nuclear translocation of activated ERK through a direct protein–protein interaction, thereby providing a direct mechanism for hScrib regulation of the ERK signalling cascade.

Results

Loss of hScrib enhances ERK nuclear localization

Previous studies have shown that loss of hScrib cooperates with activated ras in the induction of invasion in MCF10A cells, an activity that seemed to be related with Scribble's ability to downregulate ERK signalling (Dow *et al.*, 2008). We therefore first wanted to investigate whether hScrib could potentially affect ERK signalling in human keratinocytes. To do this, we generated a series of HaCaT cell lines in which hScrib levels had been ablated using short hairpin RNA targeting vectors (Massimi *et al.*, 2008). Cells were then either left untreated or exposed to osmotic shock for 30 min to enhance ERK activation. The cells were then extracted and the levels of hScrib, total ERK and activated phospho-ERK were monitored by western blotting. The results in Figure 1a show a modest level of constitutively active ERK in HaCaT cells. However, in the hScrib knockdown cells there is a marked increase in the levels of phospho-ERK in both cell lines and this increases further after osmotic shock. These results show that hScrib can contribute to the regulation of the ERK signalling cascade in human keratinocytes.

Activated ERK has been shown to translocate to the nucleus (Chen *et al.*, 1992; Gonzalez *et al.*, 1993; Lenormand *et al.*, 1993; Treisman, 1996; Fukuda *et al.*, 1997; Khokhlatchev *et al.*, 1998; Pouyssegur *et al.*, 2002). We therefore investigated whether there was a change in the pattern of ERK localization in HaCaT cells when hScrib expression levels were reduced. To do this, immunofluorescence analysis of total and phospho-ERK expression was carried out on the control and shScrib cell lines, and the results obtained are shown in Figure 1b. As can be seen, loss of hScrib (Figure 1biii) also results in a significant increase in the amount of nuclear-translocated ERK. In contrast, activated ERK seems to accumulate in Golgi-like structures in the cytoplasm of the control cells (Figures 1bi and bii), consistent with previous reports (Torii *et al.*, 2004). To verify these results, we also performed a series of transient small interfering RNA experiments, in which hScrib levels were ablated in HEK293 cells, and the levels of phospho-ERK in both total cell extracts (Figure 2a) or in the respective cellular fractions (Figure 2b) were analysed by western blotting. In both cases, loss of hScrib enhanced ERK activation and also resulted in enhanced nuclear accumulation of active phospho-ERK. These results show that one consequence of hScrib knockdown is enhanced nuclear translocation of activated ERK.

hScrib is a substrate of ERK and PKA

Having confirmed that hScrib could regulate ERK activation and nuclear translocation, we next wanted to investigate the mechanism by which this might occur. Analysis of the hScrib sequence revealed the presence of two perfect ERK-binding sites (kinase interaction motif (KIM) sites) at positions 836aa–846aa and 1396aa–1404aa (Figure 3). In addition, two potential ERK phospho-acceptor sites are correspondingly located at residues S853 and S1448, downstream of each of the two KIM sites (Figure 3).

To first investigate whether either of these two potential phospho-acceptor sites on hScrib was phosphorylated *in vivo*, we transfected cells with hemagglutinin (HA)-tagged hScrib expression plasmid and grew the cells with or without osmotic shock. The cells were then extracted, and hScrib protein was immunoprecipitated with anti-HA agarose beads. The subsequently gel-purified protein was then subjected to mass spectroscopy analysis. A summary of the phospho-peptides that were identified under the two culture conditions is shown in Figure 3. As can be seen, the N-terminal site at position S853 is phosphorylated in unstressed conditions, as is S1445 in the carboxy terminal region of the protein. Interestingly, after exposure to osmotic shock, S853 remains phosphorylated, whereas S1445 is no longer phosphorylated and the phosphorylation event occurs exclusively on S1448, just three amino acids downstream. These results show clear differential phosphorylation of hScrib *in vivo*, both before and after osmotic stress.

On the basis of these data we reasoned that ERK was a prime candidate kinase for the phosphorylation events at S853 and S1448, with the corresponding upstream KIM sites located approximately at residues 836 and 1396, respectively, whereas the phospho-site at S1445 corresponds to a potential PKA recognition site. To first confirm whether these were the responsible kinases, we analysed whether hScrib was a substrate for ERK and PKA *in vitro*. To do this, a glutathione *S*-transferase (GST)–hScrib fusion protein was purified and incubated with the purified recombinant kinases and [γ^{32}P]-ATP, and the results obtained are shown in Figure 4a. As can be seen, GST–hScrib is a good substrate for phosphorylation by both PKA and ERK1, and is only a very weak substrate for JNK and ERK2. To then determine whether the putative KIM and phospho-acceptor sites on hScrib corresponded to those identified *in vivo*, we generated a series of GST–hScrib fusion proteins that had been mutated in both the ERK KIM recognition sites, the two potential ERK phospho-acceptor sites and in the potential PKA phospho-acceptor site (Figure 4b). The purified GST proteins were then incubated with the purified kinases and [γ^{32}P]-ATP, and the results obtained are shown in Figure 4c. As can be seen, the PKA phospho-acceptor site on

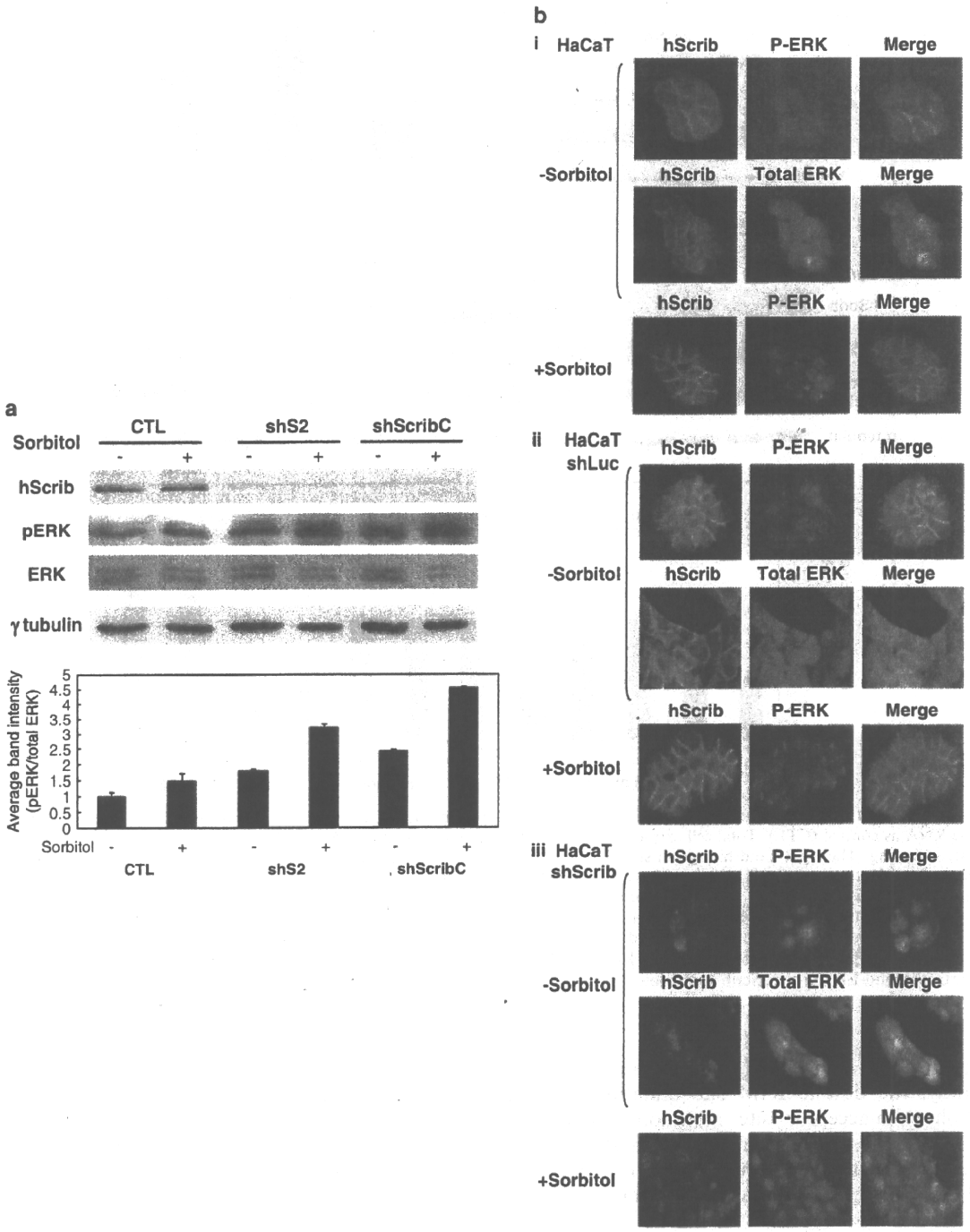

Figure 1 hScrib suppresses/downregulates the Raf/MEK/ERK pathway. (**a**) The sh-hScrib stable cell lines (S2, ScribC) and control cells (CTL) were cultured overnight and either left untreated or exposed to sorbitol for 30 min as indicated. The cells were then harvested and levels of ERK, phospho-ERK and hScrib were analysed by western blotting. γ-Tubulin was used as a loading control. The lower histogram shows the quantitative analyses of the intensities of the pERK bands from three independent experiments with s.d. indicated. (**b**) Immunofluorescent analysis of hScrib and ERK expression. HaCaT cells (i), sh-Luc control TR cells (ii) and sh-hScrib cells (iii) were grown on coverslips and then exposed to sorbitol as indicated. The cells were then fixed and double stained with the anti-hScrib antibody, the anti-phospho-ERK1/2 antibody or the anti-total ERK1/2 antibody.

GST–hScrib maps precisely to residue S1445 identified in the mass spectroscopic analysis. In the case of ERK1 the results are more complex. First, there are clearly two phospho-acceptor sites, at S853 and S1448, again corresponding to the two sites that were identified *in vivo*. Both KIM sites seem to be important for ERK1 recognition, with mutation of either site decreasing the phosphorylation to a level equivalent to that observed

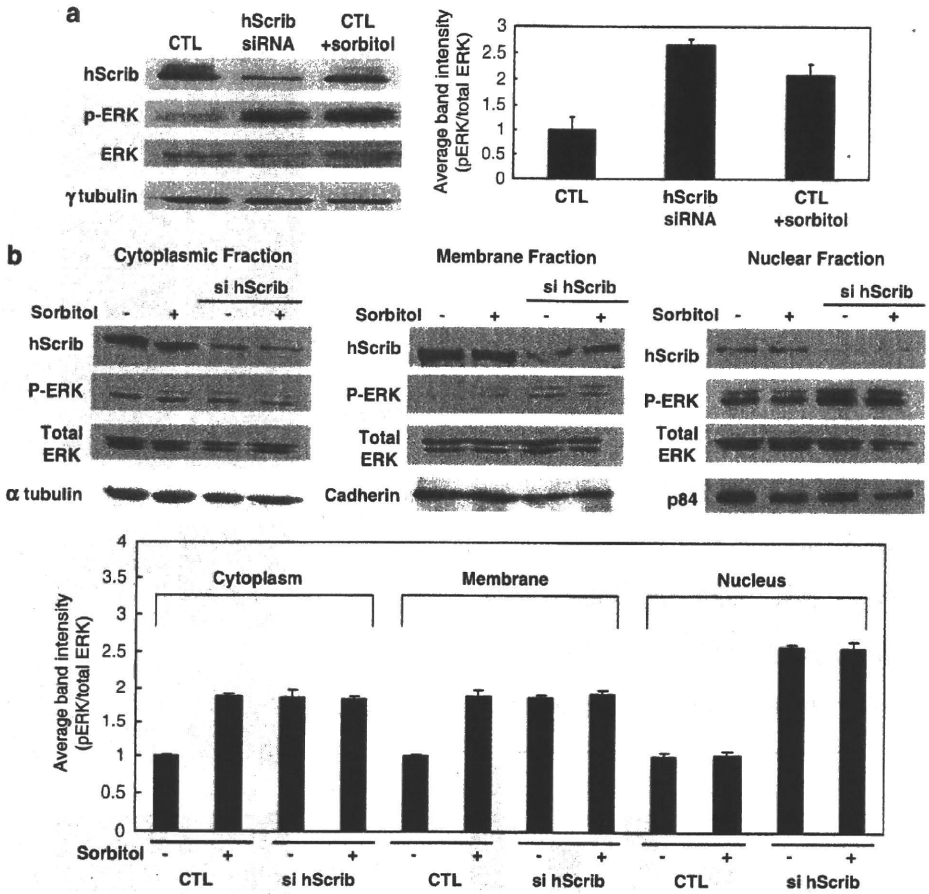

Figure 2 Loss of hScrib enhances phospho-ERK nuclear translocation. (**a**) HEK293 cells were transfected with hScrib siRNA and siLuc RNA as control (CTL). Total cell extracts were then made after 48 h, and hScrib, pERK, ERK and γ-tubulin were detected by western blotting. The right-hand histogram shows the quantitative changes in phospho-ERK/total ERK levels from a minimum of three independent assays. (**b**) HEK293 cells were transfected with hScrib siRNA and siLuc RNA as control, and then exposed to sorbitol for 20 min as indicated. Cells were fractionated into cytoplasmic, membrane and nuclear pools then phospho-ERK was then detected by western blotting. p84 was used as a loading control for the nuclear fraction, E-cadherin was used as a loading control for the membrane fraction and α-tubulin was used as the loading control for the cytoplasmic fraction. The lower histogram shows the quantitative changes in phospho-ERK/total ERK levels from a minimum of three independent assays. Note the relative increase in nuclear phospho-ERK after hScrib knockdown.

with the respective single phospho-site mutations. Most importantly, the double KIM site mutations, or the double phospho-acceptor site mutations, completely abolish ERK phosphorylation of hScrib (Figure 4c). In contrast, all of these mutants are still recognized by PKA (Figure 4d), showing that these mutations do not overly perturb the overall structure of hScrib, and further show the specificity of the assays. These results show that hScrib has two ERK docking sites and two corresponding phospho-acceptor sites, with S853 phosphorylated under normal growth conditions and S1448 being phosphorylated under conditions of osmotic stress.

hScrib regulates ERK activation through the two KIM docking sites

We next wanted to investigate whether the two identified KIM sites could actually serve as docking sites for ERK *in vitro* and *in vivo*. The GST–hScrib fusion proteins were first used in pull-down assays using the commercially purified ERK1, and levels of bound ERK1 were assessed by western blotting. The results obtained are shown in Figure 5a and show a strong direct interaction between the wild-type hScrib and ERK1. In contrast, mutation of the C-terminal KIM site largely abolishes the interaction, showing that most of the interaction is through this carboxy terminal site, although a weaker interaction is also mediated by the N-terminal KIM site. To investigate whether these sites on hScrib were also responsible for ERK binding *in vivo*, we first used the GST–hScrib fusion proteins to pull down ERK from cell extracts and then analysed by western blotting for total and phospho-ERK. The results obtained are shown in Figure 5b. As can be seen, the two KIM sites contribute to hScrib binding to ERK, although the C-terminal site seems to be the strongest site of interaction, with mutation of this single site almost abolishing ERK

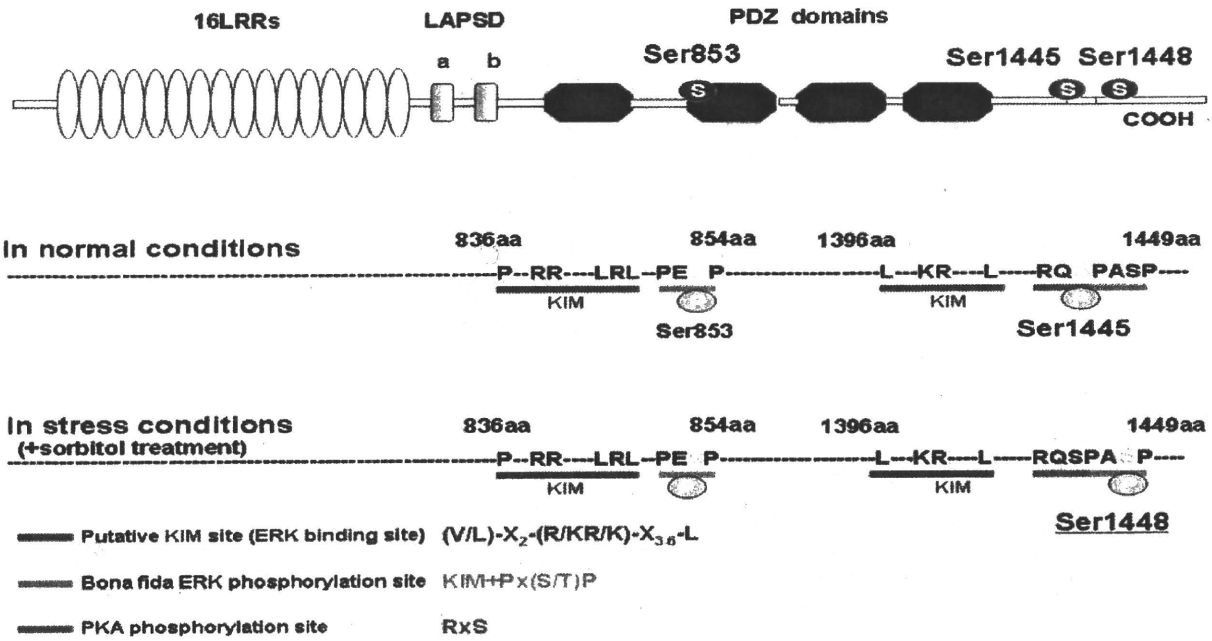

Figure 3 Identification of hScrib phospho-acceptor sites. Lysates from HEK293 cells transfected with HA-tagged hScrib either in the absence (−) or presence (+) of sorbitol for 30 min were subjected to immunoprecipitation with an anti-HA antibody. Complexes were run on SDS–PAGE and the hScrib gel slice was then subjected to mass spectrometry. Residues at S853, S1445 and S1448 were identified as phospho sites. The consensus ERK-phosphorylation motif (PESP, PASP), the consensus PKA-phosphorylation motif (RXS) (Pearson and Kemp, 1991) and the putative ERK docking site or a kinase interaction motif ((V/L)-X_2-(R/K)-(R/K)-X_{3-6}-L) (MacKenzie *et al*., 2000; Tanoue *et al*., 2000; Fantz *et al*., 2001; Zhou *et al*., 2006) are shown, in which X is any amino acid.

binding. Interestingly, when extracts are made from cells exposed to osmotic stress, there is a marked increase in the amount of ERK complexed with hScrib, suggesting that ERK activation can enhance its interaction with hScrib. We also carried out co-immunoprecipitation experiments in which HA-tagged wild-type and the ΔKIM mutant of hScrib were transfected into HEK293 cells, and then immunoprecipitated with anti-HA agarose beads. The co-precipitated ERK was then detected by western blotting. The results in Figure 5c also show that hScrib binds ERK *in vivo* in a KIM site-dependent manner and, in addition, this interaction also seems to be enhanced when the cells are exposed to osmotic stress.

The above results show that loss of hScrib enhances ERK activation and that hScrib can interact with ERK through two KIM docking sites. We next wanted to investigate whether the ability of hScrib to directly interact with ERK was responsible for its ability to downregulate ERK activation. To do this, HEK293 cells were transfected with the wild-type hScrib and the ΔKIM mutants. After 24 h, the cells were extracted and the levels of activated phospho-ERK were analysed by western blotting. The results obtained are shown in Figure 6 and show a clear downregulation in the levels of phospho-ERK when wild-type hScrib is overexpressed. In contrast, the hScrib that has the carboxy terminal KIM site mutation is no longer capable of affecting the levels of ERK phosphorylation, whereas the amino terminal KIM site mutant can downregulate phospho-ERK to levels close to those obtained with the wild-type hScrib. These results show that hScrib can directly regulate the levels of ERK activation through a direct protein–protein interaction with ERK.

To determine whether hScrib downregulation of ERK activation was physiologically relevant, we analysed the effects of the wild-type and non-ERK binding mutant of hScrib in an oncogene cooperation assay. Primary baby rat kidney (BRK) cells were transfected with human papillomavirus-16 E7 plus EJ-ras, in the presence or absence of the hScrib-expressing plasmids. After 3 weeks, the cells were fixed and stained and the numbers of colonies counted. The results obtained are shown in Figure 7 and Table 1. As can be seen, wild-type hScrib can inhibit the oncogene cooperation between E7 and EJ-ras, whereas a non-ERK binding mutant of hScrib is compromised in this activity. These results show that hScrib binding to ERK is functionally relevant in an assay of oncogene cooperation.

Discussion

Previous studies have reported many diverse functions for the hScrib protein. These include regulation of cell proliferation, cell polarity, cell migration and cell invasion in a variety of different cell types. Perhaps the most intriguing of these observations are the demonstrations that hScrib can potentially regulate MAPK signalling. Loss of hScrib was reported to enhance cell survival by inhibiting JNK-induced apoptosis in mammary tumour models of oncogene-induced

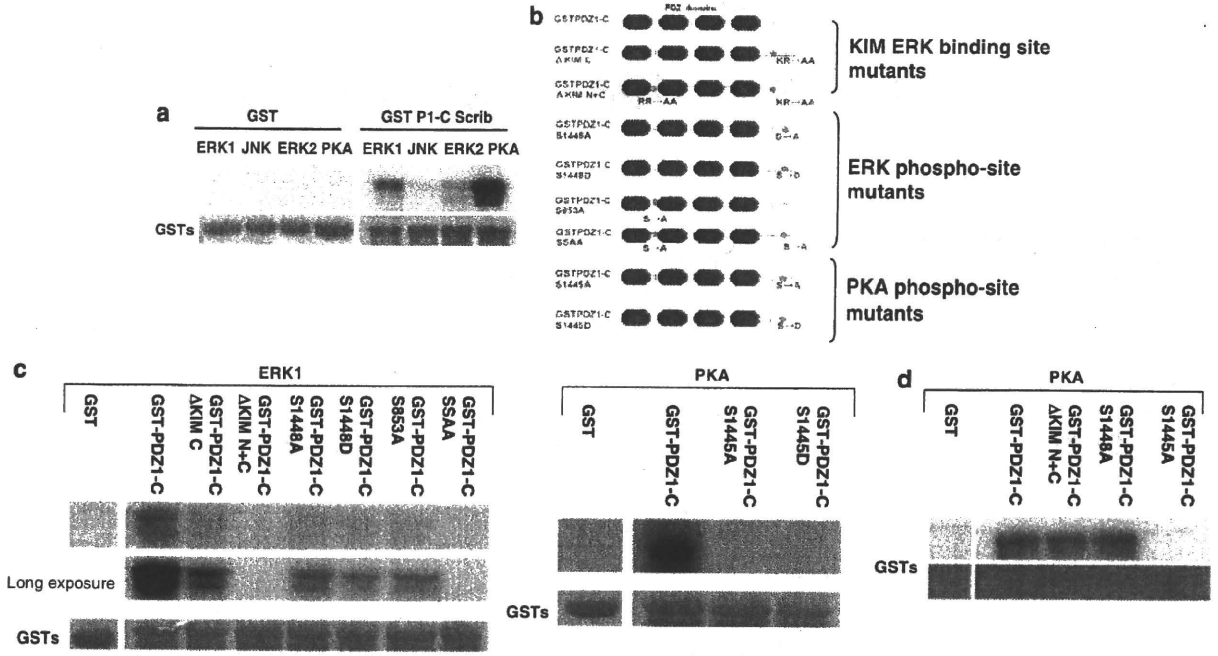

Figure 4 hScrib is a substrate for ERK1 and PKA. (**a**) The GST–hScrib fusion protein PDZ1-C (P1-C hScrib) and GST alone were incubated with purified ERK1, ERK2, JNK and PKA together with radiolabel, and after 20 min the level of phosphorylation was ascertained by SDS–PAGE and autoradiography (upper panels). The lower panels show the Coomassie protein stain of the gels. (**b**) A panel of hScrib mutants in which the ERK consensus phospho-acceptor sites (1448 and 853) and the PKA phospho-acceptor site (1445) were substituted with alanine or aspartic acid either individually or in combination. The ERK docking KIM sites (836 and 1396) sites were similarly either singly KR/AA (C terminal; ΔKIM C) or doubly RR/AA:KR/AA (N-terminal and C-terminal; ΔKIM N + C) mutated. (**c**) The wild-type and mutant hScrib fusion proteins with GST as control, were subjected to *in vitro* phosphorylation assays with ERK1 (left) or PKA (right) and then analysed by SDS–PAGE and autoradiography. The bottom panels show the Coomassie protein stains of the gels. (**d**) The wild-type and mutant hScrib fusion proteins defective in ERK1 recognition were subjected to *in vitro* phosphorylation assays with PKA and then analysed by SDS–PAGE and autoradiography. The bottom panel shows the Coomassie protein stain of the gel.

carcinogenesis (Zhan *et al*., 2008). hScrib was also reported to act upstream of ERK in the Ras/Raf/MEK/ERK signalling cascade to inhibit ERK activation and suppress Ras-induced cell invasion in breast epithelial cells (Dow *et al*., 2008). In this study, we show that hScrib regulates the ERK signalling pathway in human keratinocytes through a direct protein interaction with ERK. The consequences of this are inhibition of ERK phosphorylation and subsequent inhibition of ERK nuclear translocation.

In human skin keratinocytes, we observed that loss of hScrib expression induces an upregulation in the levels of activated phospho-ERK, providing the first indication that hScrib might also regulate ERK signalling in these cells. Most interestingly, loss of hScrib expression is accompanied by a marked accumulation of active phospho-ERK in the Golgi apparatus and in the nucleus. Although nuclear localized ERK is most likely involved in the regulation of gene expression related to cell cycle progression, Golgi accumulation may be related to the control of cell survival and cell migration, both of which have also been shown to be regulated by hScrib (Qin *et al*., 2005; Dow *et al*., 2008; Nola *et al*., 2008).

An understanding of how hScrib directly regulates ERK function has come from the identification of two ERK docking sites on hScrib. These two KIM sites are found at N- and C-terminal locations on hScrib, and both are essential for directing the interaction between ERK and hScrib, but with the C-terminal site having the strongest affinity for ERK. One of the most likely consequences of this interaction is to inhibit ERK translocation to the nucleus. However, an additional important feature is the direct inhibition of ERK activation as a result of the ability of hScrib to bind ERK. The mechanism by which this is achieved remains to be determined, although recruitment of de-activating phosphatases to the complex remains an intriguing possibility.

During the course of this analysis, we mapped three phospho-acceptor sites on hScrib. Under normal growth conditions, hScrib is phosphorylated at S853, most likely by ERK, and at S1445 by PKA. Interestingly, stimulation of MAPK by osmotic stress results in a marked loss of phosphorylation at the PKA site S1445, but a concomitant increase in phosphorylation at S1448, presumably also by ERK. Previous studies have shown that PKA phosphorylation close to a KIM site might inhibit ERK binding (Houslay and Kolch, 2000), although at present we do not know whether PKA phosphorylation can similarly affect the ability of hScrib to interact with ERK. ERK1 (p44) and ERK2 (p42)