reported an HNPCC patient who developed jejunal cancer, and a refractory pelvic mass showed significant response to FOLFOX regimen after surgical resection of the primary SBA. We observed that the lung lesions disseminated from the jejunal cancer responded to the FOLFOX-4 regimen, suggesting oxaliplatin as a promising drug for SBAs.

In conclusion, clinicopathologic correlation and immunoprofiling were very helpful in understanding the scenario of dual intestinal cancer development and for planning postoperative treatment in our patient. The incidence of SBA is gradually increasing [23], and how to treat the disease could be an emerging issue. FOLFOX-4 seems to have anticancer activity in our case, supporting further evaluation of the role of this regimen as a standard therapy for advanced SBA patients.

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C20orf20 (MRG-binding protein) as a potential therapeutic target for colorectal cancer

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BACKGROUND: Colorectal cancer is one of the most common causes of cancer death worldwide. Using cDNA microarray containing 23 040 genes, we earlier investigated gene-expression profiles in 11 colorectal cancers for the purpose of better understanding of colorectal carcinogenesis as well as development of novel diagnostic and therapeutic strategies. MRG-binding protein (*MRGBP*) or *C20orf20*, encoding a subunit of TRRAP/TIP60-containing histone acetyltransferase complex, was up-regulated in the majority of colorectal tumours.

METHODS AND RESULTS: The elevated expression of MRGBP was observed in colorectal cancer tissues by quantitative PCR as well as immunohistochemical analyses. *MRGBP* marginally expressed in normal vital organs. Notably, suppressed MRGBP expression by *MRGBP* short hairpin RNA inhibited proliferation of colorectal cancer cells. Yeast two-hybrid screening and subsequent immunoprecipitation analysis identified bromodomain containing 8 (BRD8) as an MRGBP-interacting protein. As RNA interference against *BRD8* also suppressed proliferation of colorectal cancer cells, BRD8 may be an important down-stream target of MRGBP.

CONCLUSION: These results suggest that MRGBP has an important function in proliferation of cancer cells through the regulation of BRD8 and that MRGBP should be a novel therapeutic target for colorectal cancer.

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Colorectal cancer is the second leading cause of cancer death in the United States, and its incidence rates are increasing in Japan. It is estimated that there are almost 500 000 colorectal cancer-related deaths every year in the world (Parkin, 2001). Although recent medical advances have improved the prognosis of patients with the disease, complete cure of patients with advanced tumour is far from satisfactory. In chemotherapies for advanced colorectal cancer, oxaliplatin/fluorouracil/leucovorin (FOLFOX) is an effective and well-tolerated regimen. Combination of targeted biological agents such as anti-epidermal growth factor receptor (EGFR) with FOLFOX have been reported to enhance the efficacy against EGFR-expressing metastatic colorectal cancer (Giantonio, 2006; Tabernero *et al*, 2007). This indicates that the use of rationally selected therapeutic agents will improve the treatment for advanced diseases and results in increase of cure rate and/or prolonged survival. Regarding colorectal cancer, combination chemotherapy with Bevacizumab, an inhibitor of VEGF receptor, was approved in the United States and was shown to be effective for 45% of patients with colorectal cancer and increased their 1-year survival rate from 63.4% to 74.3% (Hurwitz *et al*, 2004). However, many patients are still suffering and dying from the disease, and development of additional molecular-targeted anti-cancer drugs is a matter of pressing concern for public health.

These drugs target molecules that are expressed abundantly or exclusively in cancer cells and functioning as an indispensable factor for the growth or survival of cancer cells. For instance, Imatinib (STI571) inhibits several protein kinases such as bcr-abl fusion protein in chronic myelogenous leukaemia, and c-kit in gastrointestinal stromal tumours (O'Dwyer and Druker, 2000). Gefetinib targets the ATP cleft within the EGFR (Wakeling, 2002; Fukuoka *et al*, 2003; Gridelli *et al*, 2003; Kris *et al*, 2003). Trastuzumab is a monoclonal antibody to the HER2/neu receptor, which is overexpressed in ~30% of breast cancers (Molina *et al*, 2001). These drugs strikingly suppressed the growth of tumour cells and showed minimum cytotoxic effect in normal cells. Therefore, for the development of molecular-targeted anti-cancer drugs pinpointing cancer cells, identification of molecules that are expressed abundantly in cancer cells and clarification of their function are essential.

Molecular studies have clarified that multiple-step process has an important function in colorectal carcinogenesis, which involves activation of oncogenes such as *K-ras*, and inactivation of tumour suppressor genes such as *p53* and *APC*. In addition to these genetic changes, alteration of gene expression is involved in the carcinogenesis. Epigenetic alterations including aberrant DNA

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methylation and/or histone modification have been recently shown to participate in some of the deregulated gene expression. To unveil the molecular mechanisms of colorectal cancer and discover target molecules for the development of novel anti-cancer drugs, we analysed global gene-expression profiles of colorectal tumours by cDNA microarray analysis representing 23 040 genes (Lin *et al*, 2002). These efforts have identified a number of genes, which are frequently either up-regulated or down-regulated in the tumours compared with the corresponding non-cancerous tissues. Among the list of genes up-regulated in the tumours, we found a gene termed as MRG-binding protein (*MRGBP*), with an approved symbol of chromosome 20 open reading frame 20 (*C20orf20*), which was identified as a component of TRRAP/TIP60 histone acetyltransferase complex and shown to bind directly to MRG15 and MRGX proteins (Cai *et al*, 2003). In this report, we show, for the first time, that MRGBP expression was frequently elevated in colorectal cancer, and that it has an important function in the growth of cancer cells. These findings should contribute to a better understanding of colorectal tumourigenesis, and may serve as a starting point for the development of novel strategies for prevention and treatment of colorectal cancer.

MATERIALS AND METHODS

Cell lines and tissue specimens

A human embryonic kidney cell line, HEK293, a monkey kidney cell line, COS7, and human colon cancer cell lines, SW480 and HCT116, were obtained from the American Type Culture Collection (Manassas, VA, USA). All cells were grown in monolayers in appropriate media as follows: Dulbecco's modified Eagle's medium for HEK293 and COS7, McCoy's 5A medium for HCT116, and Leibovitz's L-15 for SW480. All media were supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution (Sigma, St Louis, MO, USA). All colorectal cancer tissues and corresponding non-cancerous tissues were obtained with informed consent from surgical specimens of patients who underwent surgery.

Isolation of RNA and quantitative PCR

Total RNA was extracted with RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturers' protocols. One microgram of total RNA was reversely transcribed for single-stranded cDNA using oligo(dT)$_{12-18}$ primer (GE Healthcare, Buckinghamshire, UK) with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative PCR was carried out using the LightCycler 480 System (Roche Diagnostics, Indianapolis, IN, USA). The probes and primers for *MRGBP* and hypoxanthine phosphoribosyltransferase1 (*HPRT1*) are as follows – *MRGBP*: forward, 5′-GGAGGAGACAGTGGTGTGG-3′, reverse, 5′-CATGTG GAAGTGTCGGTTCA-3′, and probe, Universal ProbeLibrary #39 (Roche Diagnostics); *HPRT1*: forward, 5′-TGACCTTGATTTATTT TGCATACC-3′, reverse, 5′-CGAGCAAGACGTTCAGTCCT-3′, and probe, Universal ProbeLibrary #73 (Roche Diagnostics).

Northern blot analysis

HEK293 cells transfected with pCAGGS-HA-bromodomain containing 8 (BRD8) and/or pcDNA-Myc/His-MRGBP were harvested at the indicated time points after transfection. After purification of RNA, 1 μg of poly(A) RNA was separated on a 1% agarose gel containing formaldehyde and transferred to a nylon membrane. The blot was hybridised with ^{32}P-labeled PCR product of *MRGBP* or β-actin cDNA. Human multiple-tissue northern blots were obtained from BD Biosciences (Palo Alto, CA, USA), and analysed according to the instructions of the manufacturer. The blots were autoradiographed with intensifying screens at −80°C for 5 days.

Knockdown of endogenous MRGBP expression by RNA interference

Plasmids expressing short hairpin RNA (shRNA) to *MRGBP* (psiH1BX-MRGBPs), *BRD8* (psiH1BX-BRD8), or *EGFP* (psiH1BX-EGFP) were prepared as described earlier (Shimokawa *et al*, 2003). Briefly, psiH1BX-MRGBPs and psiH1BX-BRD8 were constructed by the cloning of double-stranded oligonucleotides into psiH1BX vector and the target sequences of synthetic oligonucleotides for *MRGBP* shRNAs were as follows: 5′-GAGAAUUUGUAGCGGUUA U-3′ for shMRGBP#1, 5′-GUGACAUGGAUUAGCGCUA-3′ for shMRGBP#2, 5′-ACAAAGUCCUGACCGCAAA-3′ for shMRGBP#3, 5′-GGGAGAAGUGGUGGAAACU-3′ for shBRD8. To evaluate the knockdown effect on MRGBP and BRD8, SW480 and HCT116 cells were transfected with these shRNA constructs using Nucleofector kit (Amaxa, Gaithersburg, MD, USA), and western blotting was performed. For cell proliferation assay, psiH1BX-MRGBPs, psiH1BX-BRD8, or psiH1BX-EGFP were transfected into SW480 and HCT116 cells using FuGENE6 (Roche Diagnostics) according to the manufacturer's protocol. Transfectants were selected in appropriate concentration of Geneticin (SW480: 1.25 mg ml^{-1}, HCT116: 0.7 mg ml^{-1} for 7–9 days), and the viable cells were measured by WST-8 assay (Dojindo, Kumamoto, Japan). Control (ON-TARGET*plus* Non-Targeting pool, Dharmacon, Lafayette, CO, USA) and a mixture of four MRGBP-specific On-Targetplus siRNA oligos (5′-GAGAAUUUGUAGCGGUUAU-3′, 5′-GUGACAUGGAU UAGCGCUA-3′, 5′-ACAAAGUCCUGACCGCAAA-3′, and 5′-CAGG GAAAACCUCGGAUUA-3′) were also used for the functional analysis.

Flow cytometry

Cultured colorectal cancer cells were transfected with control or *MRBGP* siRNA (Dharmacon) for 48 h. For analysis of cellular DNA content, transfected cells were collected and fixed with 70% ethanol, and then kept at −20°C before use. Cells were incubated with 2 mg ml^{-1} RNase A at 37°C for 30 min and stained with propidium iodide (PI) at room temperature for 30 min. Assessment of apoptosis by annexin V and PI double staining was performed using Annexin V-FITC Apoptosis Detection kit (Medical & Biological Laboratories, Nagoya, Japan). Cellular DNA synthesis was evaluated by incorporation of 5-ethynyl-2′-deoxyuridine (EdU) using Click-iT EdU Flow Cytometry Assay kit (Invitrogen). Briefly, transfected cells were cultured in media containing 10 μM EdU for 30 min. The incorporated EdU and total DNA were stained with Alexa448-conjugated azide and 7-amino-actinomycin D (7-AAD), respectively. Subsequently, the cell suspensions were analysed on an FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA) using FlowJo software (Tree Star, Ashland, OR, USA).

Construction of plasmids expressing MRGBP and BRD8

The entire coding region of *MRGBP* and *BRD8* were amplified by RT–PCR using gene-specific primer sets. The primer sequences used for the amplification were 5′-TGTGAATTCGCCATGGGAGA GGC-3′ (forward) and 5′-TAACTCGAGCGTGCGGCGCCGCTT-3′ (reverse) for *MRGBP*, and 5′-ATAGAATTCTCTTCTGTCATGAGA AGTGG-3′ (forward) and 5′-ATACTCGAGTCACTTTTTCATCTT C-3′ (reverse) for *BRD8*. The cDNA products of *MRGBP* and *BRD8* were cloned into an appropriate cloning site of pcDNA3.1-Myc/His (Invitrogen) or pCAGGS-HA vector, respectively. DNA sequences of all constructs were confirmed by DNA sequencing (ABI3730, Applied Biosystems, Foster City, CA, USA).

Immunohistochemical staining using polyclonal antibody against MRGBP

We prepared histidine-tagged human MRGBP protein in bacteria, and raised rabbit antibodies specific to MRGBP by immunising rabbits with the MRGBP protein. Purification of antibodies was carried out with standard protocols using affinity columns (Affi-Gel 15, Bio-Rad, Hercules, CA, USA). Specificity of the antibodies was examined by immunoblot analysis using whole extracts from cells expressing Myc-tagged MRGBP (data not shown). Immunohistochemical staining was performed using anti-MRGBP polyclonal antibody. Paraffin-embedded tissue sections were subjected to the SAB-PO peroxidase immunostaining system according to the instructions of the manufacturer (Nichirei, Tokyo, Japan).

Immunoprecipitation and western blot analysis

COS7 and HEK293 cells were transfected with pcDNA-Myc/His-MRGBP, pCAGGS-HA-BRD8, or the combination using FuGENE6. For immunoprecipitation, the cells were lysed in 0.5% Nonidet P-40 buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl) supplemented with a Protease Inhibitor Cocktail Set III (Calbiochem, San Diego, CA, USA). The whole-cell extract was incubated with anti-Myc (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-HA antibody (Roche Diagnostics), followed by Protein G-Sepharose beads (Invitrogen) at 4°C. Proteins were separated by SDS-PAGE and immunoblot analysis was performed. Horseradish peroxidase-conjugated goat anti-mouse IgG (GE Healthcare) and goat anti-rat IgG (Santa Cruz Biotechnology) served as the secondary antibody for the ECL Detection System (GE Healthcare). To examine the endogenous interaction of MRGBP and BRD8, nuclear extract from SW480 cells was incubated with anti-MRGBP or anti-p120 (BRD8) antibody (Abcam, Cambridge, UK), followed by Protein G-Sepharose beads overnight at 4°C. After washing, these immunoprecipitants were applied for SDS-PAGE. Normal rabbit IgG (Santa Cruz Biotechnology) was used as negative control.

Immunocytochemical staining

COS7 cells were transfected with pcDNA-Myc/His-MRGBP, pCAGGS-HA-BRD8, or the combination of the two. Twenty-four hours after transfection, the cells fixed with 4% paraformaldehyde were rendered permeable with PBS containing 0.1% Triton X-100. Subsequently, the cells were covered with 3% BSA in PBS to block non-specific hybridisation, and incubated with anti-Myc or anti-HA antibody. The reaction was visualised after incubation with Alexa Fluor 488 anti-mouse or Alexa Fluor 594 anti-rat secondary antibody (Invitrogen). Nuclei were counterstained with 4′,6′-diamidine-2′-phenylindole dihydrochloride.

RESULTS

Expression of MRGBP is frequently elevated in colorectal tumours

We have earlier compared expression profiles of colorectal cancers with the corresponding non-cancerous colon tissues using cDNA microarray and identified a number of up-regulated genes in the cancer cells (Lin *et al*, 2002). In this study, we investigated a gene termed as *MRGBP* (formally *C20orf20*), because its expression was elevated in 9 out of 11 tumours in our microarray data. Reportedly, MRGBP is a subunit of a transcriptional complex of TRRAP/TIP60. Subsequent quantitative PCR confirmed its elevated expression in 10 out of the additional 15 colorectal tumours compared with their matched non-cancerous mucosa (Figure 1A). Western blot analysis also showed enhanced MRGBP expression in 10 out of the additional 14 tumours examined (Figure 1B). Multiple tissue northern blot analysis using *MRGBP* cDNA as a probe detected a 1.6 kb transcript that was readily detectable in the skeletal muscle, testis, and thyroid, whereas it showed a relatively low level of expression in important normal organs such as heart, brain, lung, liver, and kidney (Figure 1C).

To further evaluate its expression levels, we performed immunohistochemical staining using 27 colorectal cancer tissues. As a result, we observed accumulated MRGBP mainly in the nucleus of cancer cells in 20 out of the 27 tumours. However, non-cancerous epithelial cells in the adjacent mucosa of the tumours did not show accumulation of MRGBP (Figure 1D).

MRGBP confers growth-promoting effect to cancer cells

To investigate a possible function of elevated *MRGBP* expression in the proliferation of cancer cells, we prepared plasmids that express *MRGBP*- and *EGFP*-specific shRNAs with neomycin resistant gene (psiH1BX-MRGBP1, -MRGBP2, -MRGBP3, and -EGFP). Transfection of SW480 or HCT116 cells with all psiH1BX-MRGBPs significantly reduced the MRGBP expression in the cells, whereas that with control plasmid (psiH1BX-EGFP) did not affect MRGBP expression (data not shown). Cells transfected with psiH1BX-MRGBPs or psiH1BX-EGFP were cultured in media containing appropriate concentration of geneticin, and the number of viable cells was examined at day 7 or 9 after the transfection. As a result, psiH1BX-MRGBPs significantly reduced the number of viable cells compared with control plasmid (Figure 2A and B). To disclose the mechanism(s) underlying the decrease of viable cells by MRGBP knockdown, we investigated induction of apoptosis, cell cycle progression, and DNA synthesis in cancer cells treated with *MRGBP* siRNA. Knockdown of *MRGBP* did not influence significantly on population of apoptotic cells (data not shown). On the other hand, cell cycle analysis showed that treatment of HCT116 and SW480 cells with *MRGBP* siRNA significantly reduced cell population in S-phase compared with control siRNA ($35.4 \pm 2.0\%$ *vs* $17.5 \pm 1.2\%$ in HCT116, $P = 0.0002$; $29.4 \pm 1.3\%$ *vs* $26.4 \pm 0.2\%$ in SW480, $P = 0.016$). Consistently, DNA synthesis was suppressed by *MRGBP* siRNA compared with control siRNA (Figure 2C). These results suggested that *MRGBP* might have an essential function in proliferation of colorectal cancer cells through regulation of cell cycle.

Identification of bromodomain containing 8 as an MRGBP-interacting protein

To further investigate the function of MRGBP, we performed yeast two-hybrid screening and identified BRD8 (also known as skeletal muscle abundant protein (SMAP) and p120) as an MRGBP-interacting protein (data not shown). As all 32 positive clones contained the C-terminal region of BRD8, the region was likely to be responsible for the interaction. To confirm the interaction between MRGBP and BRD8, immunoprecipitation assay was performed using plasmids expressing Myc/His-tagged MRGBP (pcDNA-Myc/His-MRGBP) and HA-tagged BRD8 (pCAGGS-HA-BRD8). When COS7 cells were transfected with both pcDNA-Myc/His-MRGBP and pCAGGS-HA-BRD8, immunoprecipitation with anti-HA antibody followed by immunoblotting with anti-Myc antibody showed a single band corresponding to Myc-tagged MRGBP. Consistently, immunoprecipitation with anti-Myc antibody co-precipitated HA-tagged BRD8 (Figure 3A). We further examined endogenous interaction of MRGBP with BRD8 using nuclear extract from SW480 cells. As shown in Figure 3B (upper panels), immunoprecipitation with anti-MRGBP antibody co-precipitated endogenous BRD8. In addition, usage of anti-BRD8 antibody for immunoprecipitation also showed interacting endogenous MRGBP with BRD8 (Figure 3B, lower panels). *BRD8* has been reported to express three transcript variants. Isoform 1 of

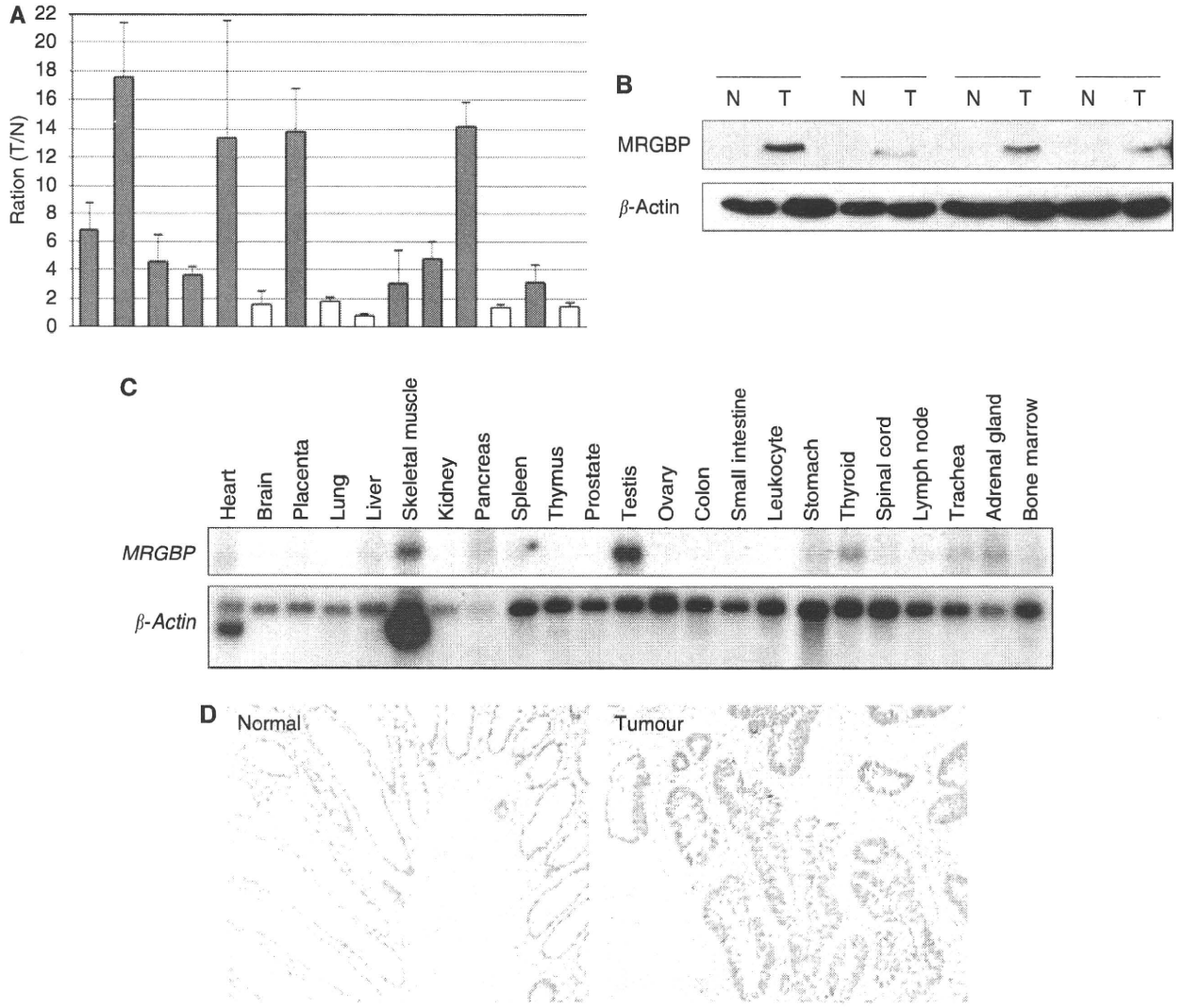


Figure 1 MRGBP is overexpressed in human colorectal tumours. (**A**) Relative expression of *MRGBP* in 15 additional colorectal tumours and the corresponding non-cancerous mucosa was analysed by real-time PCR. Quantity of *MRGBP* was normalised to *HPRT1* expression. The *y* axis indicates the ratio of mean of *MRGBP* expression in tumour to that in normal tissues. The data represents mean ± s.d. from three independent experiments. (**B**) Representative western blotting result of MRGBP in normal and tumour tissues from human colon. Expression of *β*-actin served as a control. (**C**) Multiple-tissue northern blot analysis of *MRGBP* in a panel of 23 normal human adult tissues. Expression of *β-actin* served as a control. (**D**) Representative images of immunohistochemical staining of MRGBP in normal and tumour tissues from human colon. Magnification: × 100.

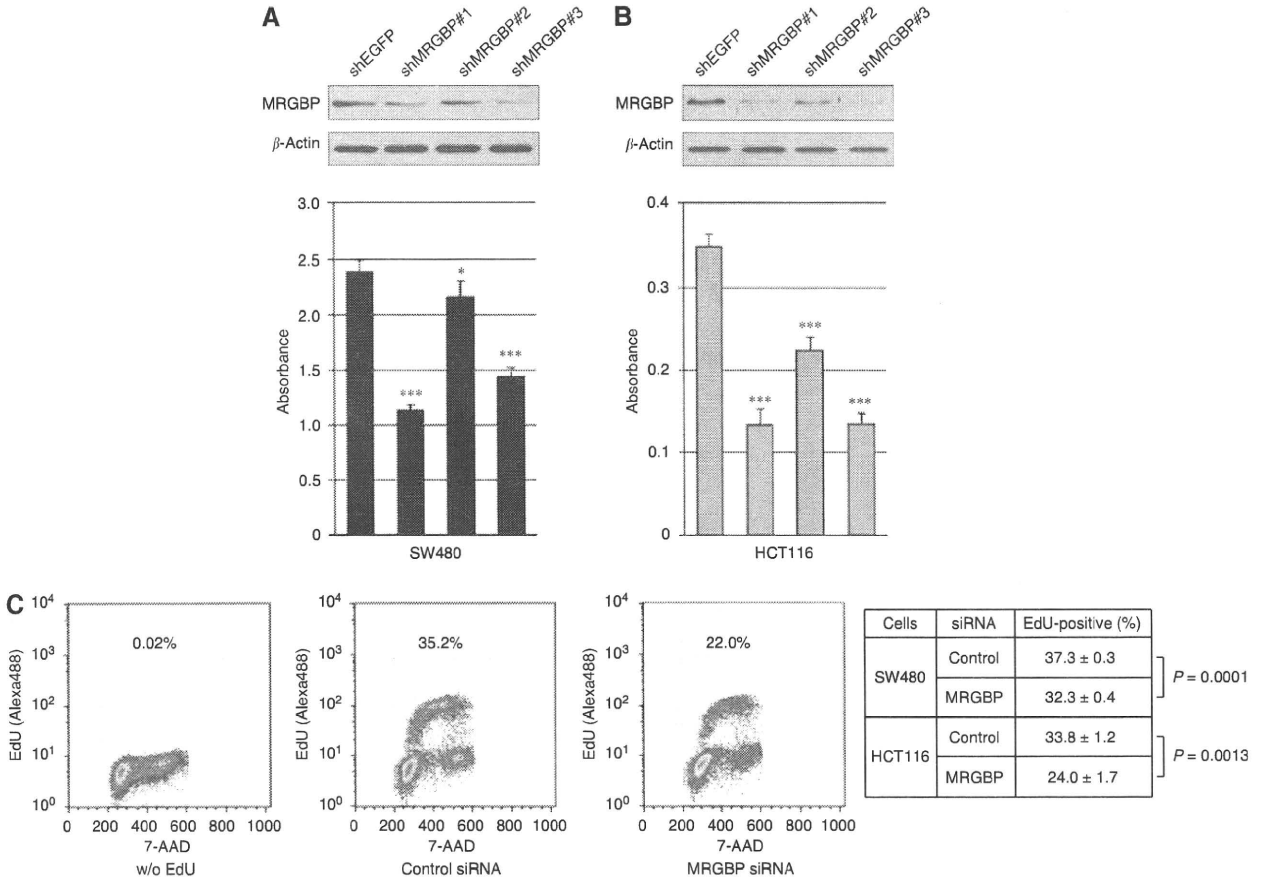
BRD8, which we cloned, represents the predominant transcript. Isoform 2 encodes a protein with a longer and different C-terminal region compared with isoform 1. Although BRD8 isoform 1 contains one bromodomain, isoform 2 contains two. We additionally performed immunoprecipitation experiment using isoform 2 expression plasmid. As a result, isoform 2 also interacted with MRGBP (Supplementary Figure 1A).

Responsible region of MRGBP for the interaction with BRD8

To address the responsible region of MRGBP for the interaction with BRD8, we prepared various deletion mutants of MRGBP (Figure 3C). Immunoprecipitation and subsequent immunoblot analysis disclosed that wild type and MRGBPΔ1, an N-terminal deletion mutant containing codons 24–204 bound with BRD8. However, MRGBPΔ2 containing codons 44–204 did not associate with BRD8. In addition, MRGBPΔ4, another deletion mutant containing codons 1–90 interacted with BRD8, whereas MRGBPΔ3 containing codons 1–76 did not (Figure 3C). These data indicated that codons 24–90 could be essential for the interaction.

MRGBP increases BRD8 protein in a post-transcriptional manner

To examine the levels of BRD8 expression resulting from the interaction with MRGBP, we carried out western blot analysis. Compared with cells expressing exogenous BRD8 alone, the presence of MRGBP markedly enhanced expression of BRD8. The induced BRD8 expression was dependent on the time of transfection, with a continuous increase up to 48 h. In contrast, the levels of BRD8 were unchanged without MRGBP (Figure 4A, upper panels). We also analysed *BRD8* mRNA in the cells by northern blot analysis. As shown in Figure 4A (lower panels), the levels of *BRD8* mRNA were not affected by MRGBP, indicating that the MRGBP-induced BRD8 protein results from post-transcriptional mechanisms. As another experiment showed that MG132, a proteasome inhibitor, greatly enhanced BRD8 protein (Supplementary



Cells	siRNA	EdU-positive (%)	
SW480	Control	37.3 ± 0.3	$P = 0.0001$
	MRGBP	32.3 ± 0.4	
HCT116	Control	33.8 ± 1.2	$P = 0.0013$
	MRGBP	24.0 ± 1.7	

Figure 2 Effect of *MRGBP* shRNA on the proliferation of colorectal cancer cells. (**A**) SW480 and (**B**) HCT116 cells were treated with *MRGBP* shRNAs or *EGFP* shRNA (control) for 48 h, and western blot analysis was performed. Expression of β-actin served as a control. Viability of cells transfected with shRNAs was measured by cell proliferation assay kit. The data represents mean ± s.d. from five independent transfections. A significant difference was determined by Student's *t*-test; *, $P<0.05$; ***, $P<0.001$, *vs EGFP* shRNA-transfected cells. (**C**) SW480 and HCT116 cells were treated with control or *MRGBP* siRNA for 48 h, and then were incubated with 10 μM EdU for 30 min. Representative flow cytometric results of HCT116 cells without EdU incorporation (left) and the cells transfected with control siRNA (middle) or *MRGBP* siRNA (right) were shown. The data represents mean ± s.d. from three independent transfections. A significant difference was determined by Student's *t*-test.

Figure 1B), the degradation might have an important function in regulating BRD8 expression. Consistent with this view, wild type and mutant MRGBP (Δ1 and Δ4) that associated with BRD8 increased BRD8 expression (Figure 3C). However, mutant MRGBP (MRGBPΔ2 and Δ3) lacking the binding ability with BRD8 did not enhance BRD8 expression (Figure 3C). To confirm the evidence that MRGBP regulates BRD8 expression, we finally knocked down endogenous MRGBP using siRNA. In complete agreement with the result of elevated BRD8 expression by MRGBP, knockdown of MRGBP substantially down-regulated BRD8 expression (Figure 4B). These results identified BRD8 as a novel down-stream target of MRGBP.

DISCUSSION

We have shown for the first time that MRGBP (C20orf20) is up-regulated in the majority of colorectal cancer, and that its elevated expression is implicated in the proliferation of cancer cells. In addition, we have discovered that MRGBP associates with BRD8. Analysis of TRRAP/TIP60 complex by mass spectrometry identified a number of components including MRGBP (Cai *et al*, 2003). Consistent with our finding, BRD8 was also included in the complex (Cai *et al*, 2003). As all positive yeast clones contained bromodomain in the C-terminal region, bromodomain might be responsible for the binding. Three alternatively spliced forms of BRD8 transcripts have been reported, and all forms include one or two bromodomains at their C-terminal. The predominant variant of transcripts encodes p120 (BRD8 isoform 1), a coactivating factor for thyroid hormone receptor (Monden *et al*, 1997). Interestingly, p120 was also found to interact with PPARγ/RXR heterodimer on PPAR-response elements in the presence of the ligand (Monden *et al*, 1999), suggesting that p120 should be involved in transcriptional regulation. BRD8 isoform 2 contains two bromodomains and has a longer C-terminus compared with p120. Variant 3 encodes SMAP (or BRD8 isoform 3), which was isolated as a highly expressed transcript in skeletal muscle (Nielsen *et al*, 1996). However, the function of SMAP has not been clarified. In addition to isoform 1, we confirmed that MRGBP associates with isoform 2. As isoform 3 shares the same bromodomain with isoform 1, MRGBP should also interact with isoform 3.

In this study, we also examined co-localisation of MRGBP and BRD8 by immunocytochemical staining (Supplementary Figure 2). Consistent with the data of immunohistochemical analysis of MRGBP, exogenous MRGBP protein was accumulated in the nucleus of COS7 cells (Supplementary Figure 2A). On the other hand, BRD8 was mainly localised in the cytoplasm (Supplementary Figure 2B). Interestingly, when COS7 cells were transfected with both plasmids expressing BRD8 and MRGBP, BRD8 accumulated in the nucleus and co-localised with MRGBP (Supplementary Figure 2C). These data implicate that MRGBP alters subcellular

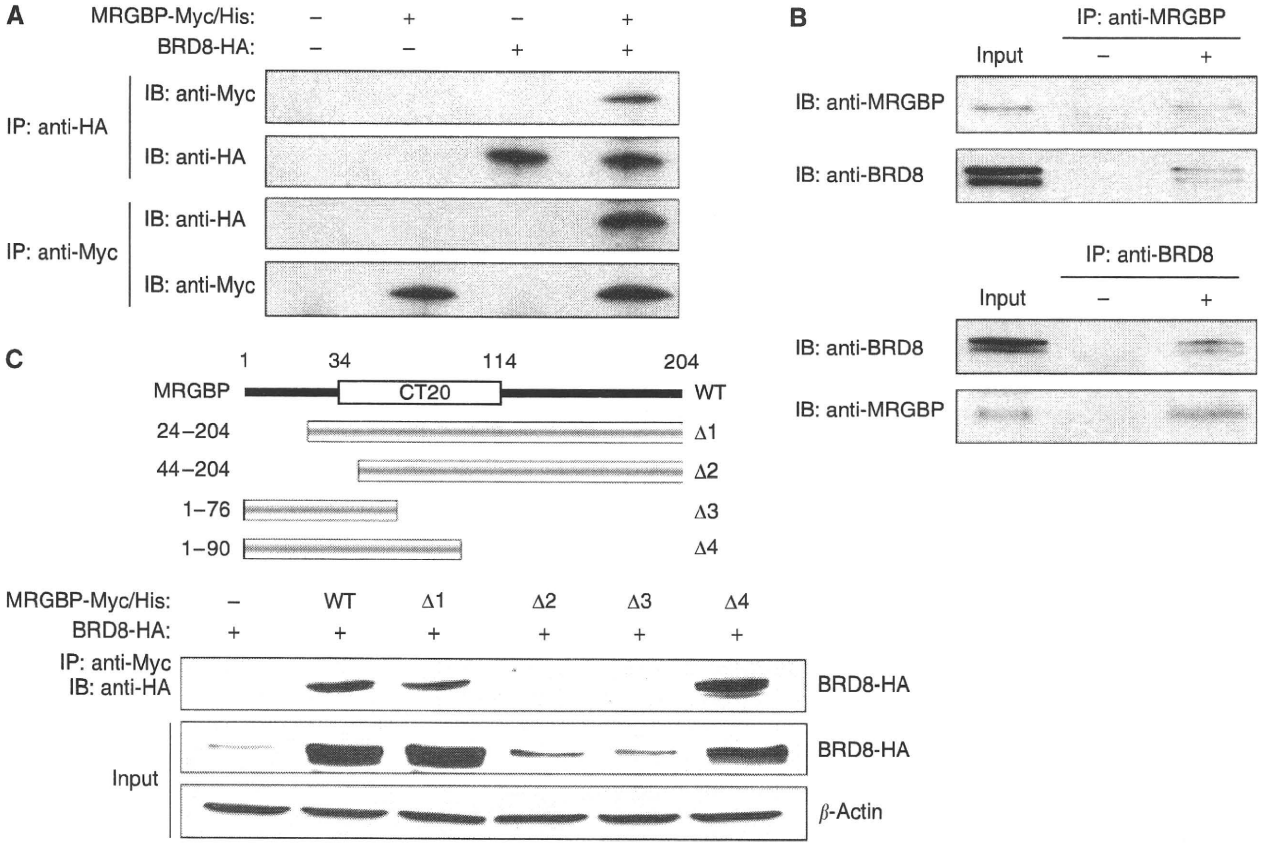


Figure 3 Interaction between MRGBP and BRD8. (**A**) COS7 cells were transfected with pcDNA-Myc/His-MRGBP, pCAGGS-HA-BRD8, or the combination of the two. Extracts from these cells were immunoprecipitated with anti-HA (upper two panels) or anti-Myc antibody (lower two panels). (**B**) Nuclear extract from SW480 cells were immunoprecipitated with anti-MRGBP (upper two panels) or anti-BRD8 antibody (lower two panels). Western blot analysis was performed using the indicated antibodies. (**C**) Interaction of wild type or the deletion mutants of MRGBP with BRD8. HEK293 cells were transfected with HA-tagged BRD8 plasmid and wild type or several deletion mutants of Myc-tagged MRGBP plasmids. Extracts from these cells were immunoprecipitated with anti-Myc antibody and then immunoblotted with anti-HA antibody (upper panel). Expressions of HA-tagged BRD8 and β-actin were shown in the middle panel and the lower panel, respectively.

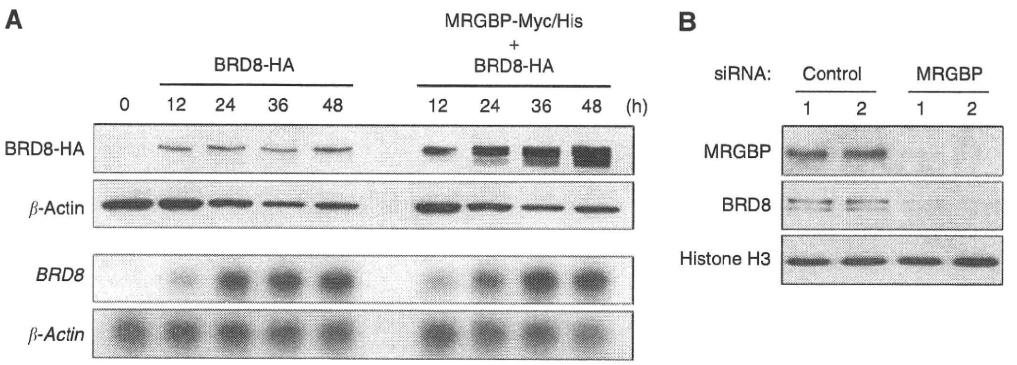


Figure 4 MRGBP increases expression of BRD8 protein in a post-transcriptional manner. (**A**) HEK293 cells were transfected with pcDNA-Myc/His-MRGBP and/or pCAGGS-HA-BRD8. After transfection, the cells were harvested at the indicated time points, and western blot analysis was performed (upper two panels). Expression of β-actin served as a control. Simultaneous northern blot analysis using *BRD8* or *β-actin* cDNA as a probe was performed (lower two panels). Expression of *β-actin* served as a control. (**B**) Effect of knockdown of MRGBP on BRD8 expression. HCT116 cells were transfected with *MRGBP*-specific or control siRNA for 72 h. Nuclear extracts were isolated, and western blot analysis was performed using the indicated antibodies. Expression of Histone H3 served as a control.

localisation of BRD8 and that it increases BRD8 expression in a post-transcriptional manner. As proteasome inhibitor MG132 strikingly augmented BRD8 expression, BRD8 protein is likely to be easily degraded in the proteasome (Supplementary Figure 1B). Taken together, these data suggest that MRGBP may regulate the stability of BRD8. We also found that the interaction of MRGBP with BRD8 is essential for the MRGBP-induced BRD8 accumulation (Figure 3C). Therefore, MRGBP may participate in the shuttling of BRD8 into the nucleus in which proteolysis machinery is inactive. Furthermore, to address the function of BRD8 on cell proliferation, we conducted cell proliferation experiment using *BRD8* shRNA construct. Treatment of HCT116 cells with *BRD8* or control shRNA showed that proliferation of HCT116 cells was significantly reduced by *BRD8* shRNA compared with control shRNA (Supplementary

Figure 2D). Therefore, BRD8 may have an important function for cell proliferation as a down-stream target of MRGBP in cancer cells. Although this hypothesis should be investigated in future studies, our findings have uncovered a novel function of MRGBP that is a member of the TRRAP/TIP60 complex.

In summary, the expression of MRGBP is enhanced in the majority of colorectal cancers, and its expression is associated with the growth of cancer cells. Interaction of MRGBP with BRD8 is probably a key for determination of MRGBP function in cancer cells. Our findings will be helpful for the profound understanding of colorectal carcinogenesis and may contribute to the development of novel anti-cancer drugs.

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Identification of SPARC as a candidate target antigen for immunotherapy of various cancers

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To establish efficient anticancer immunotherary, it is important to identify tumor-associated antigens (TAAs) directing the immune system to attack cancer. A genome-wide cDNA microarray analysis identified that secreted protein acidic and rich in cysteine (*SPARC*) gene is overexpressed in the gastric, pancreatic and colorectal cancer tissues but not in their noncancerous counterparts. This study attempted to identify HLA-A24 (*A*2402*)-restricted and SPARC-derived CTL epitopes. We previously identified H-2K^d-restricted and SPARC-derived CTL epitope peptides in BALB/c mice, of which H-2K^d-binding peptide motif is comparable with that of HLA-A24 binding peptides. By using these peptides, we tried to induce HLA-A24 (*A*2402*)-restricted and SPARC-reactive human CTLs and demonstrated an antitumor immune response. The SPARC-A24-1$_{143-151}$ (DYIGPCKYI) and SPARC-A24-4$_{225-234}$ (MYIFPVHWQF) peptides-reactive CTLs were successfully induced from peripheral blood mononuclear cells by *in vitro* stimulation with these two peptides in HLA-A24 (*A*2402*) positive healthy donors and cancer patients, and these CTLs exhibited cytotoxicity specific to cancer cells expressing both SPARC and HLA-A24 (*A*2402*). Furthermore, the adoptive transfer of the SPARC-specific CTLs could inhibit the tumor growth in nonobese diabetic/severe combined immunodeficient mice bearing human cancer cells expressing both HLA-A24 (*A*2402*) and SPARC. These findings suggest that SPARC is a potentially useful target candidate for cancer immunotherapy.

Studies on anticancer immunotherapy have been widely carried out because of its potential benefits.[1] In recent years, a number of tumor-associated antigens (TAAs) have been identified in nearly every human cancer. These TAAs have been evaluated in clinical trials, and encouraging results have been observed in some of them.[2,3] The development of highly quantitative assays for measurement of antigen-specific T cells allows a precise assessment of the endogenous tumor-reactive T-cell response in patients.[4] Such clinical studies indicate that the selection of target antigens is crucial to establish efficient anticancer immunotherapy.

Recently, cDNA microarray technologies have been developed, and an analysis of the gene expression profiles of cancer and normal cells has made it possible to determine an effective approach for the identification of the TAAs.[5–7] We previously analyzed the gene expression profiles of gastric cancers by

Key words: SPARC, tumor-associated antigen, cDNA microarrays, cancer immunotherapy, CTL, HLA-A24

Abbreviations: CTL, cytotoxic T lymphocyte; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunosorbent spot; DC, dendritic cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; HLA, human leukocyte antigen; IL, interleukin; IFN-γ, interferon-γ; mAb, monoclonal antibody; Mo-DC, monocyte-derived dendritic cell; NOD/SCID mice, Nonobese diabetic / severe compromised immunodeficient mice; NOG mice, NOD/Shi-scid IL2rγ^{null} mice; PBMCs, peripheral blood mononuclear cells; PHA, phytohemagglutinin; RT-PCR, Reverse Transcription-PCR; SPARC, Secreted Protein Acidic and Rich in Cysteine; TAA, tumor-associated antigen.

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using the genome-wide cDNA microarray consisting of probes for 20,340 genes and found that secreted protein acidic and rich in cysteine (*SPARC*) gene was overexpressed in diffuse-type gastric cancer tissues at higher levels in comparison with adjacent normal gastric tissues.[8] The levels of expression of *SPARC* in pancreatic cancer and colorectal cancer tissues were also higher than those of normal counterparts. On the other hand, the expression of *SPARC* among the normal tissue specimens was detected only in limited tissue specimens at very low levels. Therefore, we evaluated the possibility of SPARC as a target antigen for anticancer immunotherapy.

SPARC, also known as osteonectin or BM-40, has been characterized as a nonstructural matricellular glycoprotein. Its primary role is to mediate cell-matrix interactions, and SPARC has an important role in wound repair and tissue remodeling. It also plays an important role in counter-adhesion of cells, cell proliferation, cell migration and angiogenesis.[9–11] Many studies have shown that SPARC is overexpressed in various cancers including gastric cancer,[12–14] pancreatic cancer[15–18] and colorectal cancer,[19–22] and SPARC is a marker for poor prognosis in different cancer types.[13,19,23–30] SPARC may also play a crucial role in conditioning of the tumor microenvironment.[31,32] SPARC overexpression by tumor cells and surrounding stromal cells results in extensive remodeling and redistribution of extracellular matrix components promoting cell invasion and metastasis.[18] SPARC may also contribute to the invasive and metastatic properties of tumor cells, because expression of SPARC is strongly correlated with the upregulation of *Snail* and downregulation of *E-cadherin*, which promote the epithelial-mesenchymal transition.[33,34] These findings suggest the possibility that SPARC-targeting immunotherapy may be effective in attacking cancer cells and also surrounding stromal cells.

Our previous study identified H-2K^d-restricted SPARC-derived epitopes in BALB/c mice.[35] The peptide binding motif of H-2 K^d is similar to that of HLA-A24, and the amino acid sequences of the previously identified SPARC-derived epitopes carry the binding motif of HLA-A24. This study examined whether these epitope peptides could induce the human SPARC-derived cytotoxic T lymphocytes (CTLs) from the peripheral blood mononuclear cells (PBMCs) of healthy donors and cancer patients.

Materials and Methods

Mice

Seven-week-old female BALB/c mice (H-2^d), and Six-week-old female nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were purchased from Charles River Japan (Yokohama, Japan). Six-week-old male NOD/Shi-scid IL2rγ^{null} (NOG) mice were purchased from the Central Institute for Experimental Animals (Kawasaki, Japan). The mice were maintained at the Center for Animal Resources and Development of Kumamoto University, and they were handled in accordance with the animal care guidelines of Kumamoto University.

Patients, blood samples and tumor tissues

The clinical research using PBMCs from the donors was approved by the Institutional Review Board of Kumamoto University, Kumamoto, Japan. The blood samples, cancer tissues and adjacent noncancerous tissues were obtained during routine diagnostic procedures after obtaining formal written informed consent from the patients in Kumamoto University Hospital. Blood samples were also obtained from healthy donors after receiving their written informed consent. Tissue samples for RNA extraction and blood samples were anonymized, numbered at random and then stored at −80°C until use. All patients and healthy donors were of Japanese nationality.

cDNA Microarray analysis

Laser microbeam microdissection, extraction of RNA and T7-based RNA amplification and profiling of gene expression by cDNA microarray analysis was done, as described previously.[8] The raw data of microarray analysis is available on request to Professor Y. Nakamura (Institute of Medical Science, University of Tokyo). Primary diffuse-type gastric cancers and corresponding noncancerous gastric mucosa were obtained from 20 Japanese patients who underwent a gastrectomy, after obtaining a formal informed written consent from the patients. Poly(A)$^+$ RNAs isolated from human brain, lung, heart, liver, kidney, pancreas, spleen, thyroid, thymus, stomach, small intestine, colon, skeletal muscle, adipose tissue, spinal code, trachea, mammary gland, bone marrow, ovary, uterus, prostate, testis, lymph node, placenta, fetal brain, fetal lung, fetal liver and fetal kidney were used as targets for the cDNA microarray analysis.

Reverse transcription-polymerase chain reaction

Reverse transcription-polymerase chain reaction (RT-PCR) analysis of the normal tissue, cancer tissues and cancer cell lines was done as described previously.[36] *SPARC* gene-specific PCR primers were designed to amplify the fragments of 374 bp; *SPARC* PCR primer sequences were sense, 5′-CGAAGA GGAGGTGGTGGCGGAAAA-3′ and antisense, 5′-GGTTG TTGTCCTCATCCCTCTCATAC-3′.[37]

Lentiviral gene transfer

A lentiviral vector-mediated gene transfer was performed as described.[38] Briefly, 17 μg of CSII-CMV-RfA and CSIIEF-RfA self-inactivating vectors carrying *SPARC* cDNAs and 10 μg of pCMV-VSV-G-RSV-Rev and pCAG-HIVgp were transfected into the 293T cells using lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). The viral particles was suspended and added to 5×10^4 TE10 cells or SW620 cells. The expression of the transfected *SPARC* gene was confirmed by ELISA and Western blot analysis.[37,39]

Immunohistochemical staining

Immunohistochemical examinations of tissue samples to detect SPARC protein were performed as described

previously.[37,40] The staining of tissue sections with anti-human SPARC monoclonal antibody (mAb; AON-5031; Santa Cruz Biotechnology, CA) was done at a concentration of 2 μg/ml by incubating at 4°C for overnight. We used isotype-matched control mouse IgG1 and also AON-5031 mAb preincubated with 50-times molar excess of recombinant SPARC protein for control experiments.

Induction of SPARC peptide-reactive human CTLs

Human SPARC-derived peptides (purity >95%), SPARC-A24-$1_{143-151}$ (DYIGPCKYI) and SPARC-A24-$4_{225-234}$ (MYIFPVHWQF), with binding motifs for HLA-A24 (*A*2402*) were purchased from Any Gen (Korea).

PBMCs were isolated from the heparinized blood of HLA-A24 (*A*2402*)-positive Japanese patients with gastric, pancreatic and colorectal cancers or healthy donors by means of Ficoll-Conray density gradient centrifugation. Peripheral monocyte-derived dendritic cells (Mo-DCs) were generated as described previously.[5,6] CTL induction was performed according to a procedure described previously with only slight modification.[41] Briefly, autologous DCs were pulsed with 50 μg/ml candidate peptides in the presence of β2-microgloblin (4 μg/ml; Sigma-Aldrich, Tokyo, Japan) for 2 hr at 37°C in AIM-V (Gibco-Invitrogen, Tokyo, Japan). DCs were then irradiated (40 Gy) and washed with AIM-V. On Day 1, 1×10^5 peptide-pulsed DCs/well were plated on 24-well plates and cultured with 2×10^6 $CD8^+$ T cells in 2 ml of AIM-V containing 2% heat-inactivated autologous plasma and supplemented with human recombinant interleukin (IL)-7 (5 ng/ml; Wako, Osaka Japan). On Day 2, human recombinant IL-2 (PeproTech, Rocky Hill, NJ) was added to each well at a concentration of 10 units/ml.

On Day 7, 5×10^5 phytohemagglutinin (PHA)-blasts were pulsed with 50 μg/ml of candidate peptides, irradiated (100 Gy), washed once and then added to each well. On Day 8, human recombinant IL-2 was added to each well at a concentration of 50 units/ml. The peptide stimulation using PHA blasts as stimulator cells was repeated every 7 days. During CTL induction, the cells were fed with fresh AIM-V medium containing 2% heat-inactivated autologous plasma and supplemented with human recombinant IL-2 (50 units/ml) every 3 to 4 days. On Day 28, the frequency of T cells producing interferon (IFN)-γ on stimulation with the antigenic peptides and cytotoxic activity of the T cells was assessed by enzyme-linked immunosorbent spot (ELISPOT) assay and ^{51}Cr release assay, respectively. PHA-blasts were obtained as described previously.[42] The methods for IFN-γ ELISPOT assay[42] and ^{51}Cr release assay[43] were described previously.

CTL responses against cancer cell lines

The SPARC peptide-derived CTLs were cocultured with each of the cancer cells, or the peptide-pulsed C1R-A*2402 cells, as a target cell (5×10^3/well) at the indicated effector/target ratio and a standard 6 hr ^{51}Cr release assay was done as described previously.[6] C1R-A*2402 is a EBV-transformed human B lymphoblastoid cell line expressing very low levels of HLA class I molecules other than HLA-A24 and transfected with the expression vector of HLA-A24 (*A*2402*) gene.[44] The blocking of HLA-class I, or HLA-DR, was done as described previously.[7,43]

In vivo priming of SPARC-derived peptide-reactive CTLs by using NOG mice

The method of *in vivo* priming of SPARC-derived peptide-reactive CTLs by using NOG mice was described previously.[42] Briefly, $CD8^+$ T cells (1×10^7) were separated from PBMCs of healthy donors were mixed with autologous DCs (5×10^5), preloaded with SPARC peptide and intraperitoneally (i.p.) injected into NOG mice. Seven days after the injection, DCs (5×10^5) pulsed with antigenic peptide were i.p. injected again. Fourteen days after the first injection, spleen cells of NOG mice were harvested and treated with erythrocyte lysis buffer (0.83% ammonium chloride/20 mM Hepes, pH 7.2) for 1 minute and washed. To amplify *in vivo*-primed SPARC peptide-specific CTLs in NOG mice, $CD8^+$ T cells were isolated from the spleen cells and coculture with the SPARC peptide loaded and X-ray-irradiated (100 Gy) PHA blastic cells. Approximately 2×10^6 $CD8^+$ T cells and 1×10^6 PHA blastic cells were cultured in a well of 24 well-plate in AIM-V containing 2% heat-inactivated autologous plasma and human recombinant IL-2 (100 units/ml) for 6 days.

Adoptive immunotherapy model

Experimental adoptive immunotherapy was carried out as described previously.[6] Briefly, 164 cells (melanoma cell line, $SPARC^+$, $HLA\text{-}A24^+$, 4×10^6) positive for both HLA-A24 and endogenous SPARC were inoculated subcutaneously (s.c.) into the right flank of the NOD/SCID mice. Seven days after the tumor inoculation into the mice, when the tumor sizes became some 25 mm^2, the CTLs generated from PBMCs of two healthy donors by stimulation with SPARC-A24-1 and SPARC-A24-4 peptides or those stimulated with irrelevant HLA-A24-restricted HIV peptide (ILKEPVHGV; 4×10^6), were suspended in 100 μl of PBS and were injected intravenously (i.v.). The T cells were injected i.v. two more times on Days 14 and 21. The size of the tumors was measured twice a week, and the tumor size was evaluated by measuring two perpendicular diameters using calipers.

Statistical analysis

Two-tailed Student's *t*-test was used to evaluate the statistical significance of differences in the data obtained by the ELISPOT assay and in the tumor size between the treatment groups. A value of $p < 0.05$ was considered to be significant. The statistical analysis was performed using a commercial statistical software package (SPSS for Windows, version 11.0; SPSS, Chicago, IL).

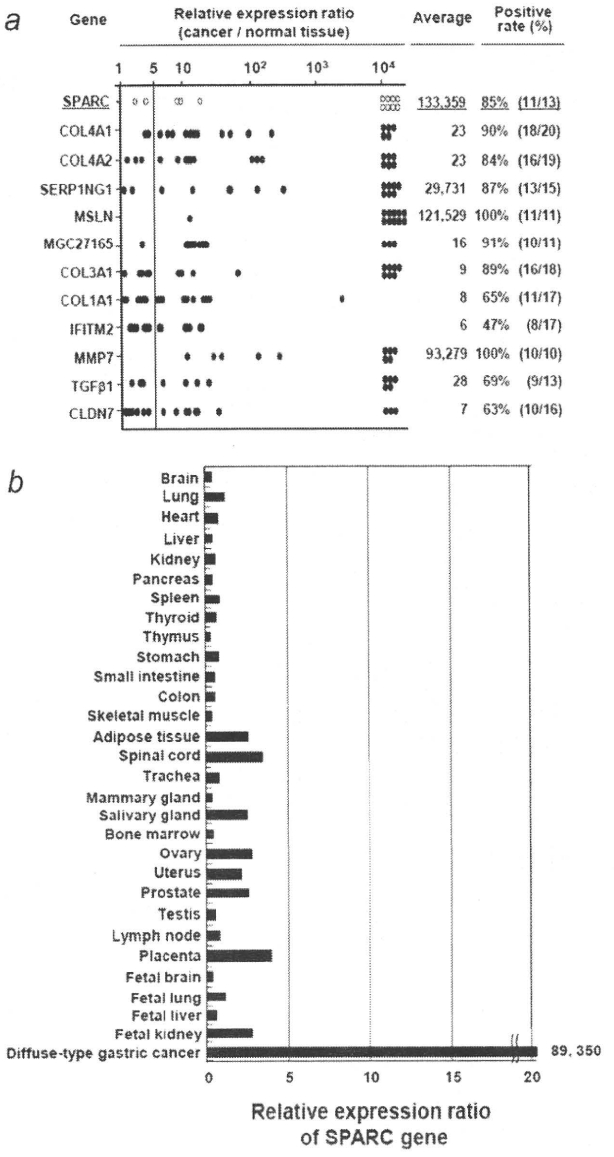

Figure 1. Markedly and frequently enhanced expression of *SPARC* mRNA in diffuse-type gastric cancer tissues based on a cDNA microarray analysis. (*a*) A list of upregulated genes in diffuse-type gastric cancer cells. These genes were overexpressed in cancer cells in comparison with their normal counterparts. The expression of *SPARC* mRNA in diffuse-type gastric cancer cells was markedly enhanced in 11 of 13 patients. (*b*) The relative expression ratio of *SPARC* gene in normal tissues based on a cDNA microarray analysis. *SPARC* gene was faintly expressed only in the spinal cord, placenta, adipose tissue and several other tissues.

Results

Overexpression of *SPARC* gene in diffuse-type and intestinal-type gastric cancers, pancreatic and colorectal cancers, revealed by cDNA microarray analysis.

The comparison of relative expression ratio of 23,040 kinds of genes among 20 cases of diffuse-type gastric cancer tissues and their adjacent normal counterparts, using cDNA microarray analysis, were reported previously.[8] These data were precisely analyzed, and 12 genes with relative expression ratios more than five times higher in diffuse-type gastric cancer tissues in comparison with its normal counterpart were identified (Fig. 1*a*). The expression of these genes was analyzed using cDNA microarray analysis in 29 kinds (including 4 embryonic tissues) of normal tissues (Fig. 1*b*). Consequently, SPARC was identified to be an adequate candidate as a target of immunotherapies for the patients with diffuse-type gastric cancer. In 11 of 13 patients, the expression of *SPARC* gene in cancer cells was more than five times higher than that in the normal counterparts (average of relative expression ratio: 133,359; Fig. 1*a*). Among the normal tissues, *SPARC* gene was faintly expressed in the placenta and spinal cord as based on cDNA microarray analysis (Fig. 1*b*). In addition, the expression level of the *SPARC* gene was also higher in the pancreatic and colorectal cancers in comparison with that in their normal counterpart, in 13 of 16 and 10 of 15 samples, respectively, based on the previous cDNA microarray analyses (Table 1; Refs. [8,45]).

Table 1. Expression of *SPARC* gene in diffuse-type gastric cancer and various malignancies investigated by cDNA microarray analyses[1]

Type of cancer	N	Positive rate[2](%)	Average of relative expression ratio
Diffuse-type gastric cancer	11/13	85	133,359
Intestinal-type gastric cancer	16/26	62	19,901
Pancreatic cancer	13/16	81	55,283
Colorectal cancer	10/15	67	15,739
Cholangiocellular carcinoma	5/25	20	2.5
Breast cancer	13/77	17	1,723
Esophageal cancer	7/64	11	1.6
Ovarian cancer	1/10	10	1.5
Prostate cancer	2/55	4	0.9
Chronic myelocytic leukemia	3/77	4	1.9
Renal cell carcinoma	3/25	12	2.6
Hepatocellular carcinoma	1/20	5	4.4
Acute myelocytic leukemia	0/54	0	0.2
Urinary bladder cancer	0/34	0	0.2
Uterine cervix cancer	0/19	0	0.6
Lung cancer	0/27	0	0.1
Osteosarcoma	0/27	0	0.8
Testicular cancer	0/13	0	0.1

[1]The data are obtained from our previous studies[8,45]. [2]The relative expression ratio (cancer/normal tissue) >5 was considered to be positive.

Expression of SPARC mRNA and protein in normal organs, cancer cell lines and gastric and colorectal cancer tissue specimens

The expression of the *SPARC* gene in normal tissue specimens at the mRNA level was analyzed using RT-PCR. A

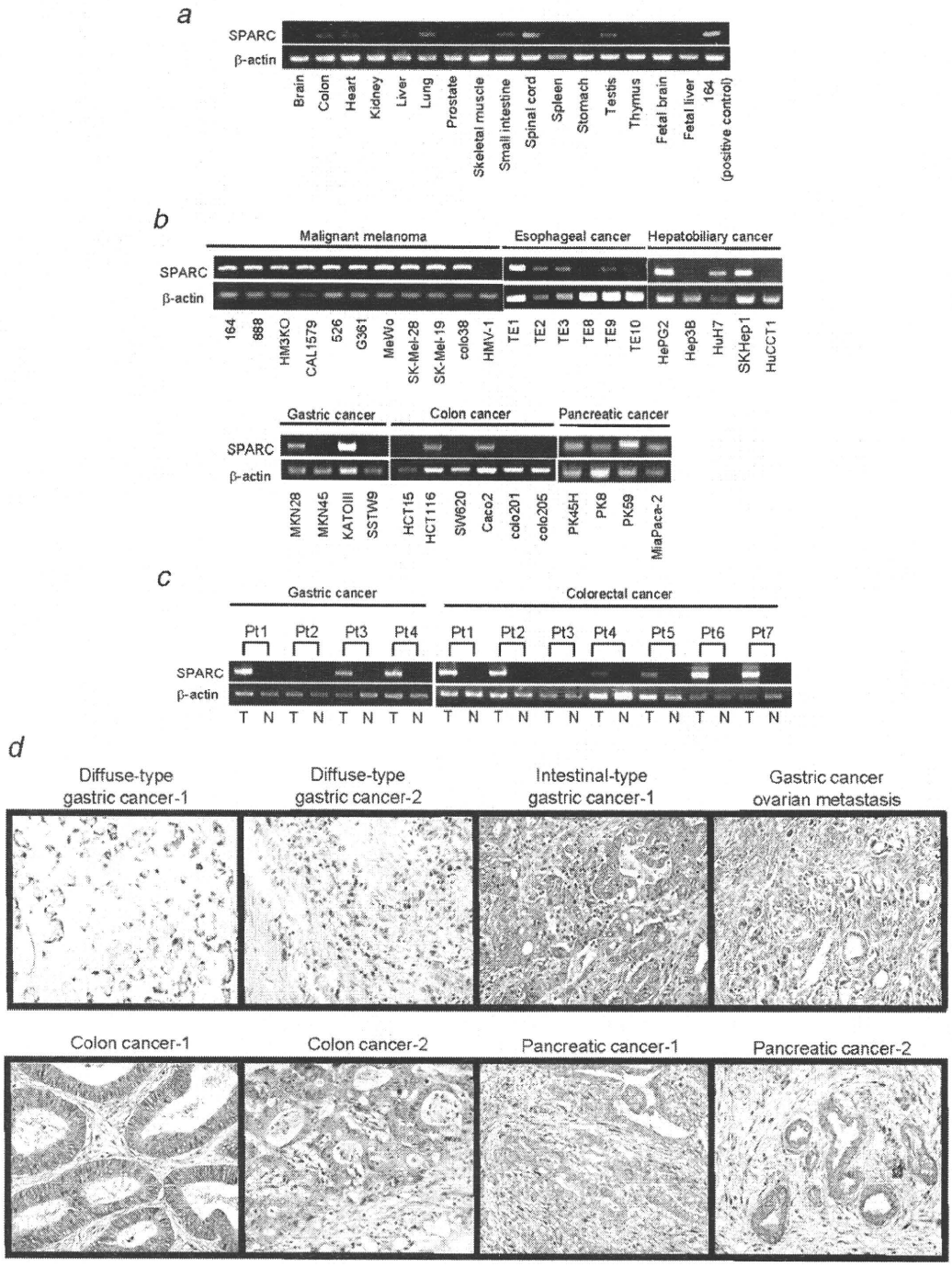

Figure 2. The analyses of *SPARC* mRNA expressed in human normal tissues, cancer cell lines and cancer tissues. (*a*) Expression of *SPARC* mRNA was investigated in various normal tissues using RT-PCR. (*b*) RT-PCR analysis of the *SPARC* expression in various cancer cell lines. (*c*) RT-PCR analysis of the *SPARC* expression in gastric and colorectal tumor tissues (T) and their normal counterparts (N). (*d*) Immunohistochemical analyses of SPARC protein in gastric, pancreatic and colorectal cancer tissues. Positive staining signals are seen as brown. Original magnification, ×100.

semiquantitative RT-PCR analysis of SPARC in the normal tissue specimens revealed it to be faintly expressed only in the spinal cord, lung, small intestine and colon (Fig. 2*a*). The expression of the *SPARC* gene was detected in the various cancer cell lines using an RT-PCR analysis. *SPARC* mRNA was expressed in 2 of 4 in gastric cancer cell lines, 2 of 5 colorectal cancer cell lines, 4 of 4 pancreas cancer cell lines, 4 of 6 esophageal cancer cell lines, 3 of 5 hepatobiliary cancer cell lines and 10 of 11 melanoma cell lines expressed *SPARC* mRNA (Fig. 2*b*).

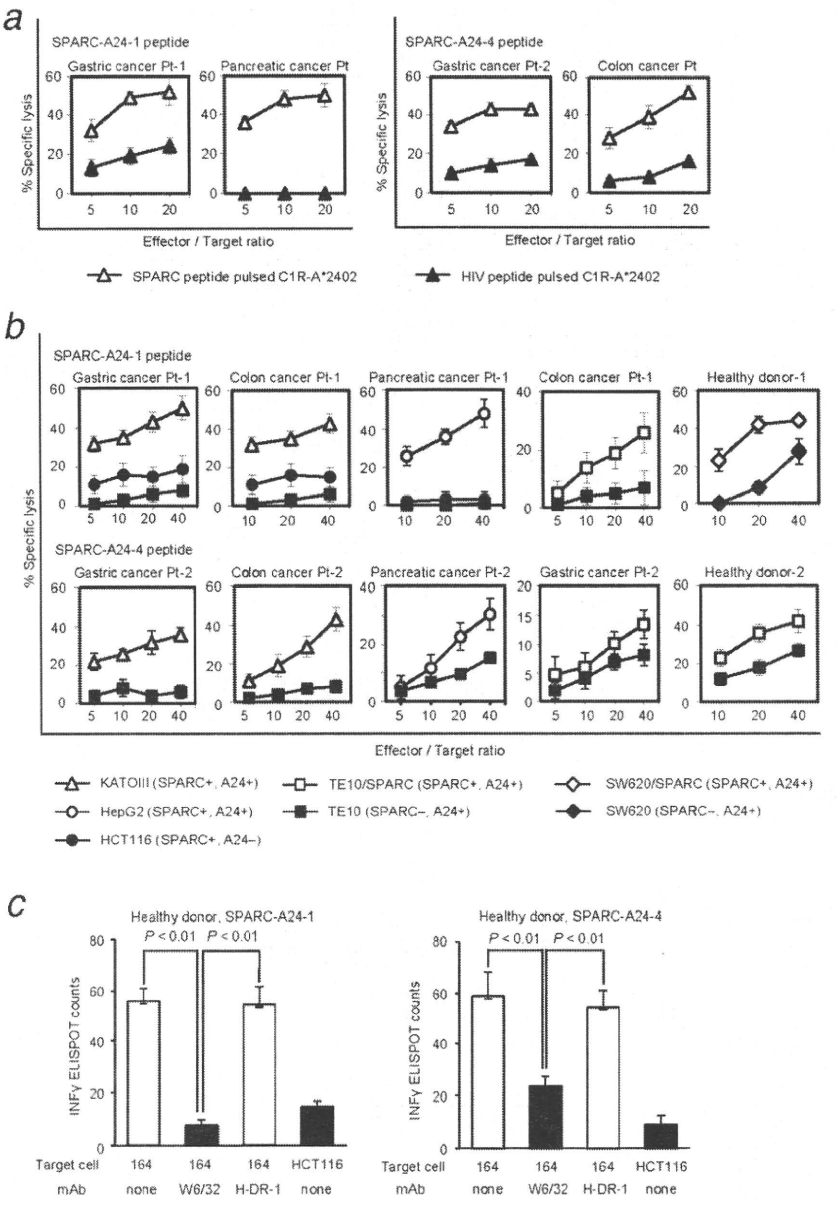

Figure 3. Induction of SPARC-specific human CTLs from the PBMCs of HLA-A24-positive healthy donors and cancer patients. (*a*) The SPARC peptide-reactive CTLs were generated from the PBMCs of HLA-A24-positive patients with gastric, pancreatic and colorectal cancers. After three times stimulation with autologous Mo-DCs and PHA blasts pulsed with the SPARC-A24-1 (left) or SPARC-A24-4 (right) peptide, the cytotoxicity of the CTLs against C1R-A*2402 cells, pulsed with each peptide or irrelevant HIV peptide, was detected by a standard 6 hr ^{51}Cr release assay. (*b*) Cytotoxicity of the SPARC-A24-1-reactive CTLs (top) and the SPARC-A24-4-reactive CTLs (bottom) directed against the SPARC^{+} HLA-A24^{+} human gastric cancer cell line KATOIII and hepatocellular carcinoma cell line HepG2, SW620/SPARC, a SPARC^{-}HLA-A24^{+} human colon cancer cell line SW620 transfected with the human SPARC gene, and TE10/SPARC, a SPARC^{-}HLA-A24^{+} human esophageal squamous cell carcinoma cell line TE10 transfected with the human *SPARC* gene, but not to SPARC^{-}HLA-A24^{+} SW620, TE10, nor SPARC^{+} HLA-A24^{-} human colorectal cancer cell line HCT116. The SPARC-reactive CTLs generated from the PBMCs of HLA-A24-positive healthy donors, gastric, pancreatic and colorectal cancer patients exhibited cytotoxicity to KATOIII, HepG2 SW620/SPARC and TE10/SPARC, but not to SW620, TE10 or HCT116. (*c*) Inhibition of cytotoxicity by anti-HLA class I mAb. After the target cells, 164, HLA-A24^{+} human melanoma cell line were incubated with anti-HLA class I mAb (W6/32, IgG2a) or anti-HLA-DR mAb (H-DR-1, IgG2a), respectively, for 1 hr, the CTLs generated from the PBMCs of healthy donors by stimulation with SPARC-A24-1 (left) or SPARC-A24-4 (right) peptide were added.

Subsequently, the expression of the *SPARC* gene was analyzed in the surgically resected cancer tissues and their adjacent normal tissues using RT-PCR. The expression of the *SPARC* gene was detected in 3 of 4 gastric cancer tissues and 6 of 7 colorectal cancer tissues, while little expression was detected in the adjacent normal tissues (Fig. 2*c*). To confirm the tumor-associated overexpression of SPARC protein, we then examined various paraffin-embedded normal tissue specimens, as well as pancreatic, gastric and colorectal cancer tissue specimens, by immunohistochemical staining. In results, positive staining was detected in gastric cancer, colorectal cancer and pancreatic cancer. The staining of SPARC was observed in the cancer cells and also in fibroblasts of the cancer stroma (Fig. 2*d*, Supporting Information Fig. 1). We also immunohistochemically analyzed fibroblasts and smooth muscle cells with anti-αSMA antibody and also endothelial cells with anti-CD31 antibody, using the same tissue sample that we used for staining with anti-SPARC antibody (Supporting Information Fig. 2).

SPARC protein was not detected in the normal brain, spinal cord, lung, liver, kidney, spleen, stomach, small intestine, colon, pancreas and skin, whereas the staining signal was observed in testis (Supporting Information Fig. 3). We confirmed the specificity of anti-SPARC mAb by Western blotting analysis. As shown in Supporting Information Figure 4, signals with the antibody were observed specifically in the SPARC-transfectant TE10 and SW620 cells but not in mock-transfected cells.

Induction of SPARC-reactive and HLA-A24-restricted CTLs from PBMCs of healthy donors and cancer patients

To identify the SPARC-derived and HLA-A24-restricted CTL epitopes, we selected a total of 4 different candidate 9- or 10-amino acid peptides that were expected to have a higher binding affinity to HLA-A24 (*A*2402*) by the HLA Peptide Binding Predictions in the BIMAS software program (BioInformatics and Molecular Analysis Section, Center for Information Technology, NIH, Bethesda, MD). The H-2K^d-binding peptide motif is comparable with that of HLA-A24-binding peptide. Thus, we previously examined CTL response of BALB/c mice to these SPARC-derived epitopes. As a result, we found that SPARC-A24-1$_{143-151}$ (DYIGPCKYI) and SPARC-A24-4$_{225-234}$ (MYIFPVHWQF) elicited high magnitude of CTL response in BALB/c mice in an H-2K^d-restricted manner.[35] Therefore, we attempted to generate SPARC-specific CTLs from the PBMCs of healthy donors and various cancer patients positive for HLA-A24 by stimulation of PBMCs with SPARC-A24-1 and SPARC-A24-4 peptides. The CD8$^+$ T cells sorted from the PBMCs were incubated with the autologous Mo-DCs and PHA blasts pulsed with each of the peptides. After stimulations with these cells, the cytotoxic activity of the T cells against the peptide-pulsed C1R-A*2402 cells was examined by a ^{51}Cr-release assay (Fig. 3*a*) and an IFN-γ ELISPOT assay (data not shown). The CTLs induced from the PBMCs of a healthy donors (data not shown), gastric and pancreatic and colon cancer patients exhibited cytotoxic activity against the C1R-A*2402 cells pulsed with SPARC-A24-1 or SPARC-A24-4 peptide, but not to the C1R-A*2402 cells loaded with an irrelevant HLA-A24 restricted HIV-derived peptide. These results indicate the peptide-specific cytotoxicity of the CTLs.

Subsequently, we analyzed whether these CTLs were able to kill human cancer cell lines expressing both SPARC and HLA-A24 or not. As shown in Figure 3*b*, the SPARC-reactive CTLs established from PBMCs derived from healthy donors and cancer patients by stimulation with SPARC-A24-1 peptide exhibited cytotoxicity to KATOIII (SPARC$^+$, HLA-A24$^+$), HepG2 (SPARC$^+$, HLA-A24$^+$), SW620/SPARC (HLA-A24$^+$ SW620 cells transfected with *SPARC* gene) and TE10/SPARC (HLA-A24$^+$ TE10 cells transfected with *SPARC* gene). All of these cells were positive for both HLA-A24 and endogenous or transgene-derived SPARC. On the other hand, the CTLs did not kill TE10 (SPARC$^-$, HLA-A24$^+$), SW620 (SPARC$^-$, HLA-A24$^+$) and HCT116 (SPARC$^+$, HLA-A24$^-$), which were negative for either HLA-A24 or SPARC. Similarly, the CTLs stimulated with SPARC-A24-4 peptide had specific cytotoxicity to the cancer cells expressing both SPARC and HLA-A24 molecules, but not to the cells that were negative for either of them.

Collectively, these data indicate that CTLs reactive to either of these two peptides killed only the cells expressing both SPARC and HLA-A24, thus suggesting that SPARC was most likely processed and presented by HLA-A24 on the surface of these cancer cells.

To confirm that the induced CTLs recognized the target cells in an HLA class I-restricted manner, the mAb against HLA class I (W6/32) was used to block the recognition by the CTLs. In this experiment, the anti-HLA class I antibody could markedly inhibit the IFN-γ production stimulated with 164 cells in an ELISPOT assay of the CTLs generated by stimulation with SPARC-A24-1 peptide, with statistical significance (Fig. 3*c* left, $p < 0.01$). Similarly, the anti-class I antibody could markedly inhibit the IFNγ production stimulated with 164 cells in an ELISPOT assay of the CTLs generated from PBMCs by stimulation with SPARC-A24-4 (Fig. 3*c*, right; $p < 0.01$). These results clearly indicate that these induced CTLs recognized the target 164 cells expressing SPARC in an HLA class I-restricted manner.

In vivo priming of SPARC-peptide specific CTL in NOD/Shi-scid IL2rγ^{null} (NOG) mice

We recently developed a method to analyze *in vivo* priming of human CTLs by Mo-DC pulsed with antigenic peptides, based on xenotransplantation of human immune cells into immunocompromised NOG mice, which are totally devoid of intrinsic functional lymphocytes.[42] In this study, we used this system to investigate the *in vivo* priming of human CTL with the SPARC-derived peptides. On Day 1, we i.p. injected CD8$^+$ T cells derived from HLA-A24 positive healthy donors along with autologous Mo-DCs pulsed with SPARC-peptide into NOG mice. A boost immunization was done by i.p. injection of SPARC-peptide pulsed Mo-DCs on Day 8. The spleen cells of NOG mice were harvested on Day 15 and cocultured with

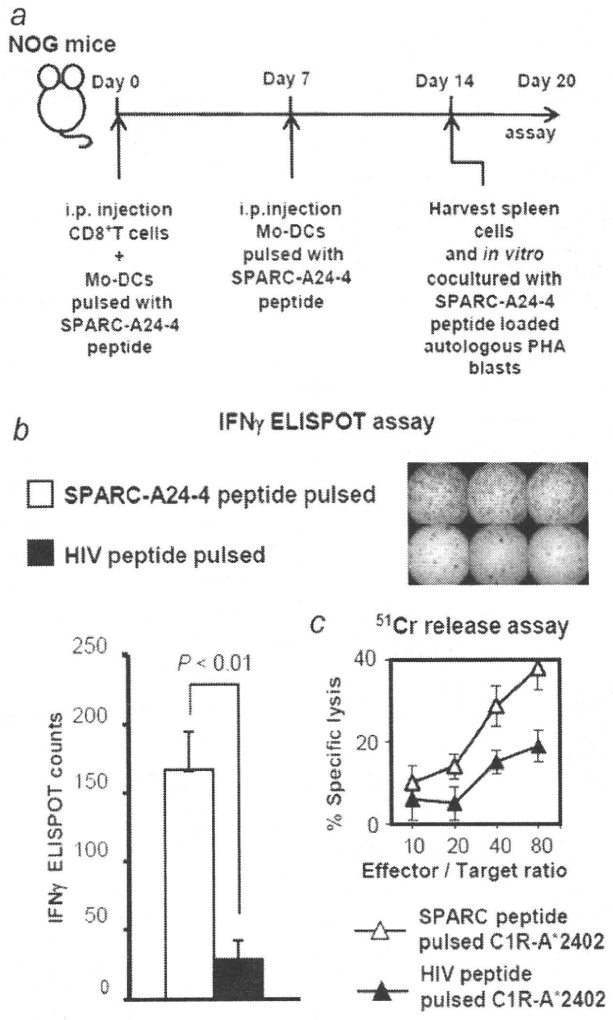

Figure 4. *In vivo* priming of SPARC-peptide specific CTLs in NOD/Shi-scid IL2rγ^{null} (NOG) mice. (*a*) A schema of *in vivo* sensitization and assay of SPARC-A24-4-specific CTLs in NOG mice. Purified CD8$^+$ T lymphocytes sorted from PBMCs were i.p. injected together with SPARC-A24-4-peptide pulsed DCs into NOG mice on Day 0. For boosting, the SPARC-A24-4peptide-pulsed DCs were i.p. injected again on Day 7. The spleen cells were recovered from the mice on Day 14 and cocultured with PHA blasts prepared from the same donor and preloaded with SPARC-A24-4peptide. After 6 days, cultured cells were recovered and analyzed. (b) The photograph images of ELISPOT assay and quantified spot counts of the SPARC-A24-4-reactive CTLs recovered from *in vivo* priming and the amplification culture against the SPARC-A24-4-peptide-loaded target cells or irrelevant peptide pulsed target cells. (*c*) The cytotoxicity of these cells analyzed by the ^{51}Cr release assay. These assays were done twice with similar results. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

PHA blasts supplemented with human recombinant IL-2. On Day 20, peptide-specific immune response was evaluated by ELISPOT assay and ^{51}Cr release assay (Fig. 4*b*). The SPARC-A24-4-reactive CTLs were successfully induced by *in vivo* priming in NOG mice (Fig. 4*b* and 4*c*).

In vivo antitumor activity of adoptively transferred SPARC-induced human CTLs in NOD/SCID mice

The therapeutic efficacy of the SPARC-reactive CTLs was assessed by inoculation into mice bearing established human tumors expressing SPARC. We inoculated s.c. 164, a human melanoma cell line positive for both SPARC and HLA-A24, into NOD/SCID mice. The intracytoplasmic staining of SPARC in 164 melanoma cells is shown in Supporting Information Figure 5. After 7 days, when the tumors grew to about 5 × 5 mm in size, CTLs generated by stimulating the CD8$^+$ T cells derived from HLA-A24 positive healthy donors with SPARC-A24-1 and SPARC-A24-4 peptides or control T cells stimulated with irrelevant HIV peptide by the same procedure were injected i.v. The control HIV peptide-stimulated CD8$^+$ T cells did not exhibit cytotoxicity against the 164 cells *in vitro* (data not shown).

The tumor size of the six individual mice in each group (Fig. 5*a*) and the mean FSD of the tumor sizes in each group (Fig. 5*b*) were evaluated. The tumor size in the mice inoculated with the SPARC-stimulated CTLs was significantly smaller than that in the mice inoculated with the control HIV peptide-induced CD8$^+$ T cells or with PBS alone ($p < 0.01$), indicating an antigen-specific antitumor effect of the SPARC-reactive CTLs *in vivo*.

However, the tumor in the mice inoculated with the SPARC-reactive CTLs enlarged again several days after the last inoculation of the CTLs. The difference of tumor size between the mice inoculated with the SPARC-reactive CTLs and those inoculated with control CTLs became unclear at 2 or 3 weeks after the Day 35. This limited efficacy of the treatment might be due to a relatively short duration of the effect of SPARC-reactive CTLs on inhibition of tumor growth *in vivo*, and antitumor effect may well be prolonged by repeated inoculations of SPARC-reactive CTLs. However, this possibility remains to be examined.

Discussion

Gastric cancer is histologically classified into diffuse-type and intestinal-type.[46] The diffuse-type often metastasizes to peritoneum or lymph nodes, resulting in poorer prognosis. The incidence of diffuse type, particularly the signet-ring type, has been increasing. Therefore, the development of means for early diagnosis and therapy of this type of gastric cancer is eagerly needed.

In this study, we identified SPARC to be a promising target antigen for cancer immunotherapy. By using a laser-microdissection cDNA microarray analysis of diffuse-type gastric cancer, we revealed distinctly high expression of *SPARC* in the cancer tissues in comparison with adjacent normal tissues. The microarray and RT-PCR data showed that *SPARC* is also overexpressed in intestinal-type gastric cancer and colorectal cancer tissues, and was not expressed in their normal counterparts. These results are consistent with the publications by other groups that described an

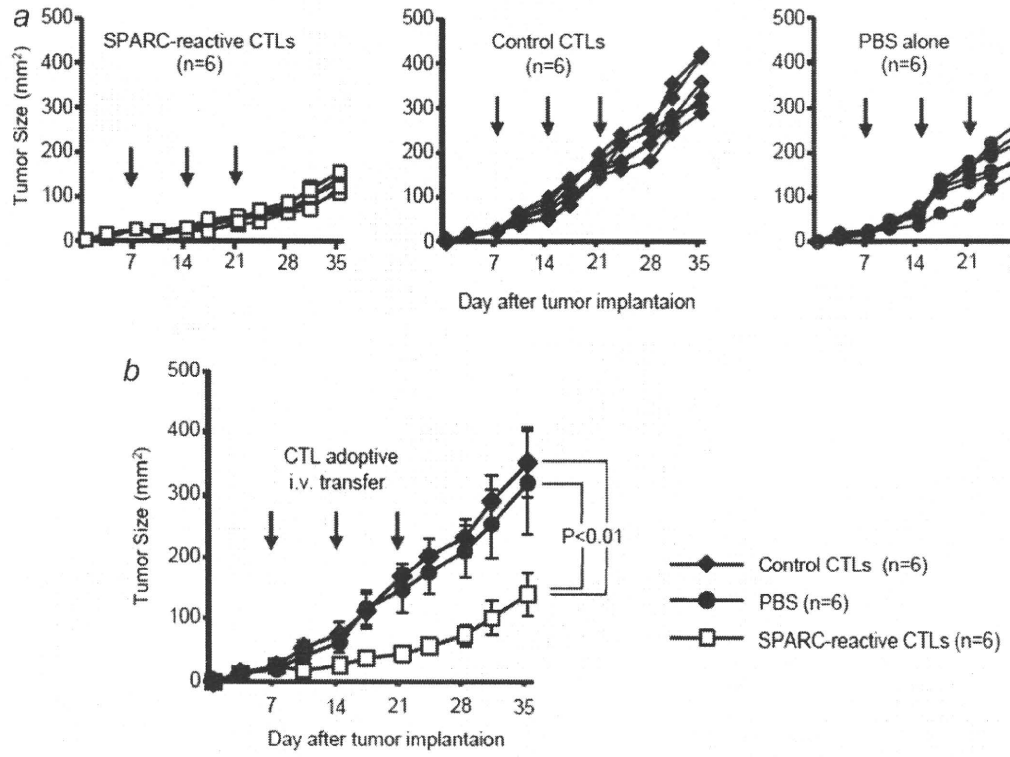

Figure 5. *In vivo* antitumor activity of the SPARC-induced human CTLs adoptively transferred into NOD/SCID mice with preestablished mass of human cancer. (*a*) Inhibition of growth of a human melanoma cell line, 164 (SPARC$^+$, HLA-A24$^+$), engrafted into NOD/SCID mice by the adoptive transfer of SPARC-reactive CTLs. When the tumor size reached some 25 mm^2 on Day 7 after s.c. tumor implantation, human CTLs (4×10^6) reactive to SPARC-A24-1 and SPARC-A24-4 peptide were inoculated i.v. On Day 14 and Day 21, the same CTL inoculation was repeated. The control CD8$^+$ T cells were prepared by stimulation of PBMCs with irrelevant HLA-A24-restricted HIV peptide. The tumor volumes in the NOD/SCID mice injected with SPARC-induced CTLs (□), control CD8$^+$ T cells (◆) or PBS alone (●) are shown. The tumor size is expressed as square millimeters. (b) Comparison of mean tumor sizes in each group of mice; bar, ±SD (n = 6 for each group).

overexpression of SPARC in a variety of human malignancies including gastric, colorectal and pancreatic cancers.[12–14,19,23–30] In our immunohistochemical analyses, SPARC was overexpressed not only in gastric, pancreatic and colorectal cancer cells but also in cancer stromal cells such as fibroblasts. These data are consistent with other reports that demonstrated the overexpression of SPARC not only in cancer cell themselves but also in the surrounding stromal cells.[15,17,19] These results suggest that successful immunotherapy targeting SPARC would possibly attack cancer tissues including both cancer cells and surrounding stromal cells.

Tumor-derived SPARC stimulates tumor progression in many types of cancers. The expression levels of SPARC correlate with the histological grade of tumor tissues.[13,24,25,27] A higher SPARC expression is associated with local tumor invasion[13,19,27,28,47]; metastasis to the lymph nodes, liver and bone[13,19,26,29]; and poor prognosis and survival.[13,19,29,30] However, precisely which cells in the cancer tissues among tumor cells, stromal cells, such as fibroblasts, and endothelial cells mainly produce SPARC is still unknown. Several reports have described that SPARC protein is overexpressed in the stromal cells of tumor tissue but is rarely expressed in cancer cells themselves.[17,28,48] Other studies showed that cancer cells do not express SPARC due to hypermethylation of the *SPARC* gene in colon cancer and pancreatic cancers.[49,50] However, the results of our RT-PCR analyses indicate frequent expression of *SPARC* mRNA in pancreatic cancer cell lines, although expression of *SPARC* mRNA in gastric and colon cancer cell lines was not so frequently observed. On the other hand, melanoma cells by themselves have been shown to express a high level of SPARC, and such increased levels are reportedly associated with an invasive phenotype *in vivo*.[23] We previously reported the presence of high levels of SPARC and glypican-3 protein in the sera of patients, and these have been proposed as markers for the diagnosis of melanoma in early stages.[37] This study suggests the possibility that SPARC may also be valuable as a target for immunotherapy of melanoma.

These findings confirmed the cytotoxicity of the SPARC-reactive CTLs not only *in vitro* by a ^{51}Cr release assay but also *in vivo* by a CTL adoptive transfer model as shown in Figure 5. Our data clearly indicated that the adoptive transfer of SPARC-reactive human CTLs into mice bearing human tumors expressing SPARC could effectively inhibit tumor growth, at least in the tumor-bearing NOD/SCID mouse model.

It is very important to investigate whether the targeting immunity toward SPARC could induce autoimmune diseases, either during or after anticancer immunotherapy. Studies of SPARC in development and aging demonstrate the importance of this matricellular protein in many tissues and throughout the normal life cycle. SPARC is pervasive in embryos and has been identified in bone, cartilage, teeth, epithelia, dermis, olfactory tract, heart, kidney, lung, testis, thyroid and gut. In the adult, expression becomes restricted to the bone, kidney, testis, hematopoietic tissue, central nervous system and cochlea.[32,51] Our results of RT-PCR analyses of *SPARC* mRNA expression in the normal tissue specimens revealed that it was faintly expressed only in the spinal cord, lung, small intestine and colon. However, SPARC protein could not be detected in these tissue specimens by immunohistochemical staining. Therefore, we considered it was safe to target immunity toward SPARC for anticancer immunotherapy. In this regard, the results of our previous study is valuable, since they show no phenomenon suggesting autoimmunity in BALB/c mice immunized with H-2K^d-restricted SPARC-derived peptide.[35]

In summary, we evaluated SPARC, a molecule overexpressed in various cancers including gastric, colorectal and pancreatic cancer, as a target antigen for anticancer immune therapy. Tumor-reactive CTLs could be induced by stimulation of PBMCs of healthy individuals or cancer patients with SPARC-derived peptides. The peptides are most likely processed from SPARC protein and presented in the context of HLA-A24 in the cancer cells. The SPARC epitopes identified in this study may, therefore, be potentially useful for a new cancer immunotherapy in these cancers.

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Leptin as a critical regulator of hepatocellular carcinoma development through modulation of human telomerase reverse transcriptase

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Abstract

Background: Numerous epidemiological studies have documented that obesity is associated with hepatocellular carcinoma (HCC). The aim of this study was to investigate the biological actions regulated by leptin, the obesity biomarker molecule, and its receptors in HCC and the correlation between leptin and human telomerase reverse transcriptase (hTERT), a known mediator of cellular immortalization.

Methods: We investigated the relationship between leptin, leptin receptors and hTERT mRNA expression in HCC and healthy liver tissue samples. In HepG2 cells, chromatin immunoprecipitation assay was used to study signal transducer and activator of transcription-3 (STAT3) and myc/mad/max transcription factors downstream of leptin which could be responsible for hTERT regulation. Flow cytometry was used for evaluation of cell cycle modifications and MMP1, 9 and 13 expression after treatment of HepG2 cells with leptin. Blocking of leptin's expression was achieved using siRNA against leptin and transfection with liposomes.

Results: We showed, for the first time, that leptin's expression is highly correlated with hTERT expression levels in HCC liver tissues. We also demonstrated in HepG2 cells that leptin-induced up-regulation of hTERT and TA was mediated through binding of STAT3 and Myc/Max/Mad network proteins on *hTERT* promoter. We also found that leptin could affect hepatocellular carcinoma progression and invasion through its interaction with cytokines and matrix mettaloproteinases (MMPs) in the tumorigenic microenvironment. Furthermore, we showed that histone modification contributes to leptin's gene regulation in HCC.

Conclusions: We propose that leptin is a key regulator of the malignant properties of hepatocellular carcinoma cells through modulation of hTERT, a critical player of oncogenesis.

Background

Obesity is an important risk factor for many types of cancer, including hepatocellular carcinoma (HCC) [1,2]. Among adipocytokines, that are the main body weight regulators, leptin, the 16-KDa nonglycosylated protein product of the Ob gene, has a central role [3,4]. It is a multifunctional peptide hormone with a wide range of biological activities including neuroendocrine function [5], angiogenesis [6,7], bone formation [8] and modulation of immune responses [9,10]. Leptin exerts its actions through its six isoforms of receptors, which are membrane spanning glycoproteins with cytoplasmic domains of varying length [11].

Leptin's signaling is thought to be transmitted mainly by the Janus-activated Kinase/signal transducers and activators of transcription (JAK/STAT) pathway [12]. Of the seven human STAT genes, STAT3 has been shown to be activated in a wide variety of human tumors and tumor cell lines and its activation is accompanied by increased expression of important cell cycle and survival regulators, such as cyclin D1, c-myc and survivin [13,14]. Many STAT3 target genes are key components of the regulation of cell cycle progression from G1 to S phase [15].

At present, a biological explanation for the association between obesity and HCC is not known. It seems that there is a strong relationship between adipocytokines,

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