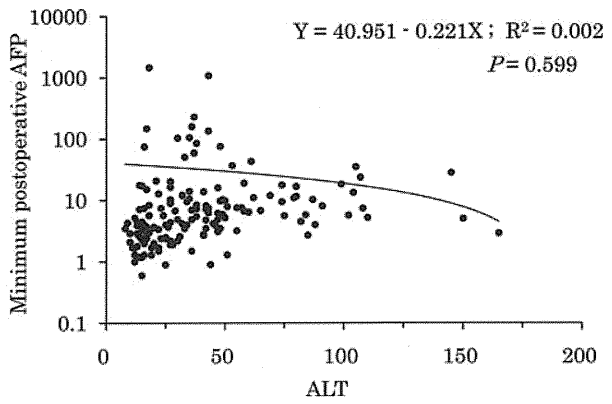

Figure 4. Correlation between minimum postoperative α-fetoprotein (AFP) and alanine aminotransferase (ALT) levels. There was no significant correlation (P=0.599).

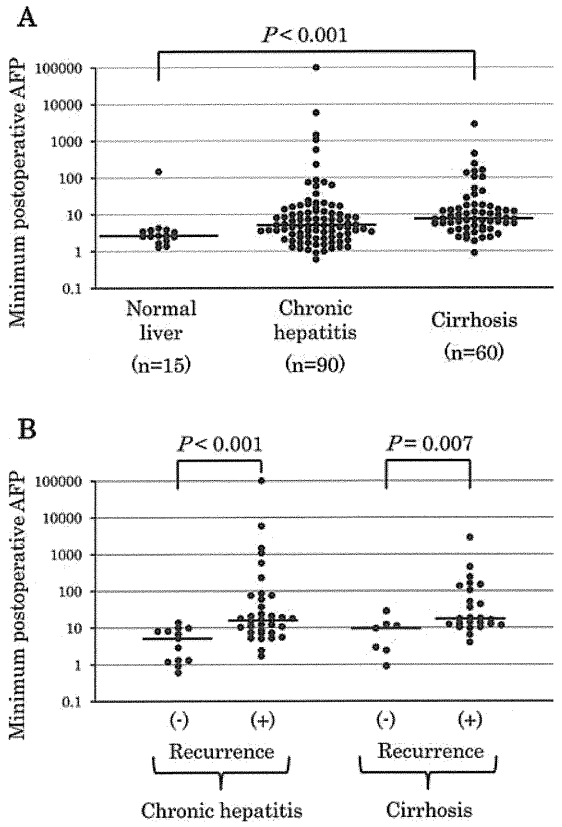

Figure 5. Correlation between minimum postoperative α-fetoprotein (AFP) and histological findings of non-cancerous lesions. (A) Patients were divided into three groups according to grade of underlying chronic liver disease. As underlying liver disease became more severe, minimum postoperative AFP level became greater (P<0.001). (B) Furthermore, patients limited to those with a positive preoperative AFP level in each group were divided into two subgroups - with and without recurrence. Minimum postoperative AFP level in patients with recurrence exceeded that in patients without recurrence in the chronic hepatitis group (P<0.001) and cirrhosis group (P=0.007). Bars are medians.

regression analysis showed no statistically significant correlation (P=0.599) (Fig. 4). To evaluate the correlation between AFP level and grade of underlying chronic liver disease, we compared minimum postoperative AFP level with histological findings of non-cancerous lesions. Fig. 5A shows minimum postoperative AFP level of the patients divided into three groups according to underlying chronic liver disease. As chronic liver disease became more severe, minimum postoperative AFP level became greater, and the difference was statistically significant by Kruskal-Wallis rank test (P<0.001). Furthermore, when patients were limited to those with a positive preoperative AFP level and the patients in each group were divided into two subgroups - those with and without recurrence, minimum postoperative AFP level of the patients with recurrence exceeded that of the patients without recurrence in the chronic hepatitis group (P<0.001) and cirrhosis group (P=0.007) (Fig. 5B).

Discussion

In this study, we demonstrated that postoperative AFP level is a useful tool for predicting HCC recurrence after curative hepatectomy. The evidence for this is that most of the patients who experienced recurrence later did not show a negative change in AFP level after curative resection. Moreover, minimum postoperative AFP level was a significant independent risk factor for recurrence. On the other hand, most of the patients who experienced recurrence later as well as those who never experienced recurrence showed a negative change in DCP level after operation, and minimum postoperative DCP was not a significant risk factor in multivariate analysis. There was no statistically significant correlation between AFP level and grade of hepatitis activity, and thus a positive level of AFP after operation might suggest a site of residual viable cancer.

Imaging modalities, including US, dynamic CT and dynamic MRI, are the gold standard for diagnosis of HCC. However, in general, since they can only detect a cancer site greater than approximately 1 cm in diameter, smaller cancer sites are missed before operation. Although intraoperative US is used to try to detect other cancer sites that have not been detected before operation, the limitations of US include its operator dependence and its poor ability to differentiate early HCC from dysplastic nodules in the cirrhotic liver. Therefore a positive level of AFP after operation might suggest a viable residual cancer site that has been undetectable by imaging modalities.

One reported problem of AFP and DCP is low sensitivity (15,16). Although measurement of two tumor markers is recommended (17-19), the sensitivity for small HCC is not yet satisfactory. However, this study showed that, when classified by preoperative level, the sensitivity of minimum postoperative AFP level was high (80.0%), whereas that of minimum postoperative DCP level was still low (19.4%). This is because AFP is superior to DCP for the diagnosis of small HCC (20). Another reported problem of AFP is low specificity because of a high false-positive rate with benign conditions such as acute and chronic active hepatitis (21-24). Several authors have demonstrated that *Lens culinaris* agglutinin-reactive α-fetoprotein (AFP-L3) can distinguish between HCC and hepatitis by detecting a sugar chain microheterogeneity (25-27). Our previous studies have demonstrated that glypican-3 (GPC3) is a novel tumor marker of HCC and is especially useful in the early stages because of its high sensitivity (28-31).

A limitation of our study is that it is difficult to determine whether an elevation of AFP is due to a residual cancer site or active hepatitis. However, our results showed no statistically significant correlation between levels of AFP and ALT, which is a well known marker of hepatitis activity (32,33). Although the histological findings of non-cancerous lesions showed a statistically significant correlation with AFP level, our results showed that patients who had higher postoperative AFP levels were most likely to experience recurrence. Moreover, not the grade of underlying chronic liver disease but postoperative AFP level was a significant risk factor for recurrence by univariate and multivariate analyses. Therefore, in most cases, a positive level of AFP after operation might mean a residual viable cancer site and not liver cirrhosis.

Generally, two different mechanisms are responsible for HCC recurrence (34). One is recurrence due to metastasis, originating from cancer cell dissemination from the primary tumor. The other is multicentric carcinogenesis of a new tumor based on underlying hepatitis or cirrhosis. However, they are not easily distinguishable (35). Instead, we distinguished between recurrence within 1 postoperative year and that after this time period as described previously (36). The result was that the majority of patients whose postoperative AFP level remained positive experienced recurrence within 1 postoperative year. Therefore, a positive level of AFP after operation suggests a site of residual viable cancer that has already occurred before operation.

In order to prevent HCC recurrence from a viable but undetectable cancer site, establishment of effective adjuvant therapy is urgently needed. We have just started a phase II clinical trial of GPC3-derived peptide vaccine for adjuvant therapy after curative operation or ablation. GPC3 is an ideal target for anticancer immunotherapy because its expression is detected specifically in most HCCs even in the early stages and is correlated with a poor outcome (37-41).

In conclusion, we have shown that minimum postoperative AFP level is an important risk factor for recurrence after curative hepatectomy. A positive level of AFP after operation might suggest a residual viable cancer site. The need for effective adjuvant therapy and close follow-up is suggested in patients with a positive postoperative AFP level. In addition, further studies will be needed to find novel useful serum markers that have better sensitivity for early detection of HCC recurrence.

Acknowledgements

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Silencing of secreted protein acidic and rich in cysteine inhibits the growth of human melanoma cells with G_1 arrest induction

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The overexpression of secreted protein acidic and rich in cysteine (SPARC) is associated with increased aggressiveness and poor prognosis in malignant melanoma. Its roles and underlying mechanisms on melanoma cell growth, however, are not fully clarified. To validate the potential of SPARC as a therapeutic target, we examined the effect of the knockdown of SPARC with SPARC-specific siRNA on the growth of human melanoma cell lines. SPARC siRNAs exerted a potent knockdown effect. Silencing of SPARC resulted in growth inhibition with G_1 arrest accompanied by accumulation of p21, a G_1 cyclin-dependent kinase inhibitor, in MeWo and CRL1579 cells. Moreover, the induction of p53 was observed in MeWo cells, but not in CRL1579 cells. Conditioned media containing SPARC from MeWo cells could not restore the growth of SPARC-silenced MeWo cells. This result suggests that intracellular SPARC, but not secreted SPARC, is involved in cell proliferation. In addition, silencing of SPARC induced apoptosis in MeWo and CRL1579 cells. Furthermore, when MeWo cells in which SPARC expression was transiently knocked down by SPARC siRNA were implanted in nude mice, the tumor growth was suppressed. Our findings suggest that SPARC contributes to cell growth and could be a potential target molecule for melanoma therapy. (*Cancer Sci* 2010; 101: 913–919)

The expression of secreted protein acidic and rich in cysteine (SPARC), a matricellular glycoprotein, is highly regulated during development, tissue repair, and remodeling.[1] SPARC interacts with several extracellular matrix components.[2] In addition, SPARC modulates growth factor activity, and regulates matrix metalloproteinase expression.[3–6] These reports suggest that SPARC regulates cell shape, proliferation, migration, and differentiation.

The level of SPARC expression is low in normal adult tissue, whereas this protein is overexpressed in a wide range of human cancers.[7–9] Some groups have reported that overexpression of SPARC is associated with aggressiveness and high potential of metastasis in various human cancers, including melanoma, breast, lung, esophagus, pancreas, and bladder cancers.[10–15] It has also been reported that its overexpression is related to poor prognosis in many cancers.[12,16,17] In most cancers, SPARC is produced in tumor stromal cells, such as fibroblasts and endothelial cells, rather than in cancer cells.[7,12,16] In contrast, the level of SPARC expression in melanoma and glioma cells is very high.[10,18]

Selective silencing of gene expression using siRNA has been evaluated to be not only a powerful research tool but also a potentially therapeutic approach to cancer.[19] It has been reported that silencing of SPARC directly inhibited the survival signaling pathway in glioma cells under serum reduced conditions *in vitro*.[20] Some studies using antisense RNA have showed that downregulation of SPARC abrogated a tumorigenic capacity in melanoma cells.[21–23] One of the reasons for this rejection appears to be the involvement of the antitumor activity of host polymorphonuclear cells. The underlying mechanism of SPARC on the growth of melanoma cells, however, has not been fully elucidated.

We have previously reported that the serum SPARC in melanoma patients was useful as a novel tumor marker for early diagnosis of melanoma,[24] and have shown the usefulness of SPARC as a target for cancer immunotherapy.[25] From these points of view, we hypothesized that SPARC might become a target molecule for cancer treatment, and examined whether silencing of SPARC with siRNA could influence cell growth in melanoma cells *in vitro* and *in vivo*. We found that silencing of SPARC in human melanoma cell lines induced G_1 cell cycle arrest and apoptosis. We herein report for the first time that silencing of endogenous SPARC by siRNA directly inhibits growth in melanoma cells.

Materials and Methods

Cell culture. Human melanoma cell lines MeWo, SK-MEL-28, and HMV-I were maintained in DMEM (Sigma, St Louis, MO, USA) containing 10% FBS (Hyclone, Logan, UT, USA). Human melanoma cell line CRL1579 was obtained from RIKEN Cell Bank, RIKEN BioResource Center (Tsukuba, Japan) and maintained in RPMI-1640 (Sigma) containing 10% FBS. All cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

RNAi and transfection. The siRNA duplexes were purchased from Qiagen (Valencia, CA, USA) (AllStars Neg. Control siRNA) and Invitrogen (Carlsbad, CA, USA) (SPARC). The siRNA sequences used were as follows: SPARC siRNA-1, 5′-AGUCACCUCUGCCACAGUUUCUUCC-3′; SPARC siRNA-2, 5′-AUACAGGGUGACCAGGACGUUCUUG-3′; and SPARC siRNA-3, 5′-AUUCUCAUGGAUCUUCUUCACCCGC-3′. Lipofectamine RNAiMax (Invitrogen) was used for the reverse transfection method following the manufacturer's protocol. For analysis of transfection efficiency, the cells were transfected with FITC-conjugated negative control siRNA (Qiagen) at 50 nM. After 24 h, the cells were analyzed using flow cytometry. Flow cytometry was carried out using a FACSCalibur (BD Biosciences, San Jose, CA, USA) and analyzed using CellQuest (BD Biosciences) and FlowJo (Tree Star, San Carlos, CA, USA) software.

Immunoblot analysis. The cell samples were lysed in appropriate amounts of lysing buffer (150 mM NaCl, 50 mM Tris [pH 7.4], 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM EDTA, and protease inhibitor tablet [Roche Applied Sciences, Penzberg, Germany]). Protein concentration was determined

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with DC protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). Cell lysates or supernatants were heat-denatured, resolved by 10% SDS-PAGE, and electrotransferred to PVDF membrane (Millipore, Billerica, MA, USA). The membranes were blocked in TBS-Tween 20 (10 mM Tris [pH 7.4], 150 mM NaCl, and 0.1% Tween 20) containing 5% non-fat milk for 2 h at room temperature and incubated overnight at 4°C with primary antibodies: anti-SPARC (Haematologic Technologies, Essex Junction, VT, USA), anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-ERK (Cell Signaling Technology, Beverly, MA, USA), and anti-β-actin (Sigma), followed by reaction with HRP-conjugated secondary antibody (Jackson Immuno Reseach, West Grove, PA, USA). In addition, polyclonal HRP-conjugated anti-p21 antibody (Santa Cruz Biotechnology) was used. The bands were visualized by ECL (GE Healthcare, Little Chalfont, UK).

Cell proliferation assay. HMV-I, MeWo, CRL1579, and SK-Mel-28 cells were transfected with SPARC siRNA or negative control siRNA at indicated concentrations, then seeded in 96-well flat bottom plates at 3×10^3, 2×10^3, 4×10^3, or 2×10^3 cells/100 μL per well, respectively. The cells were cultured in the presence of WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) (Dojindo, Kumamoto, Japan) for 3 h, followed by measurement of absorbance at 450 nm and 650 nm. For the swapping experiment, the conditioned media from siRNA-transfected MeWo cells (1×10^5 cells in six-well plate) were harvested at 72 h post-transfection, centrifuged at 1700*g* for 5 min to remove cellular debris, and stored at −80°C until use.

Cell cycle analysis and annexin V staining. For flow cytometric cell cycle analysis, the cells treated with siRNA were collected, washed with PBS, fixed in cold 70% ethanol, and stored at −20°C until staining. After fixation, the cells were washed with PBS and incubated with 50 μg/mL RNaseA (Sigma) for 30 min at 37°C, before staining with 50 μg/mL propidium iodide (Sigma). Apoptotic cells in early and late stages were detected using an annexin V–FITC Apoptosis Detection Kit from BioVision (Mountain View, CA, USA). In brief, the cells were transfected with siRNA at 10 nM. At 96 h post-transfection, culture media and cells were collected and centrifuged. After washing, cells were resuspended in 490 μL annexin V binding buffer, followed by the addition of 5 μL annexin V–FITC and 5 μL propidium iodide. The samples were incubated in the dark for 5 min at room temperature and analyzed using flow cytometry.

***In vivo* tumor experiment.** For assessment of tumor growth *in vivo*, MeWo cells were transfected with SPARC siRNA-3 or negative control siRNA. Twenty-four hours later, the cells were trypsinized, and resuspended in serum-free DMEM. Four female athymic nude mice, ages 6- to 8-weeks-old were s.c. implanted with 1×10^6 or 3×10^5 MeWo cells per 0.1 mL into the right and left flanks resulting in two tumors per mouse. The tumor volume in mm^3 was calculated by the formula: volume = $(\text{width})^2 \times \text{length}/2$. The mice were maintained under specific pathogen-free conditions. Animal experiments in this study were approved by the Animal Research Committee of the National Cancer Center Hospital East (Kashiwa, Japan).

Statistical analysis. All data are presented as the mean ± SD. The data from the WST-8 assay were statistically analyzed by one-way ANOVA followed by Dunnett's multiple comparison test or Tukey's multiple comparison test. Tumor volume between SPARC siRNA-treated cells and negative control siRNA-treated cells was compared for statistical significance using the Mann–Whitney *U*-test or Student's *t*-test. The results were considered significant when $P < 0.05$. All tests were carried out with Dr. SPSS II for Windows (SPSS Japan, Tokyo, Japan).

Results

Silencing of SPARC expression in human melanoma cell lines. We examined the knockdown effect of SPARC siRNAs on melanoma cells in this study. To assess the knockdown efficiency of SPARC, we transfected negative control siRNA or siRNAs targeted to SPARC (SPARC siRNA-1, -2, or -3) into SPARC expressing cell lines, MeWo, CRL1579, and SK-MEL-28 (Fig. 1A,B). Transfection with three SPARC siRNAs decreased the level of SPARC protein in all tested cell lines compared with negative control siRNA. This silencing effect was enhanced in a dose-dependent manner. The level of SPARC protein was not affected among the cells transfected with negative control siRNA at each concentration. Treatment with 10 nM SPARC siRNAs resulted in the robust downregulation of SPARC expression. SPARC siRNA-3 showed the strongest

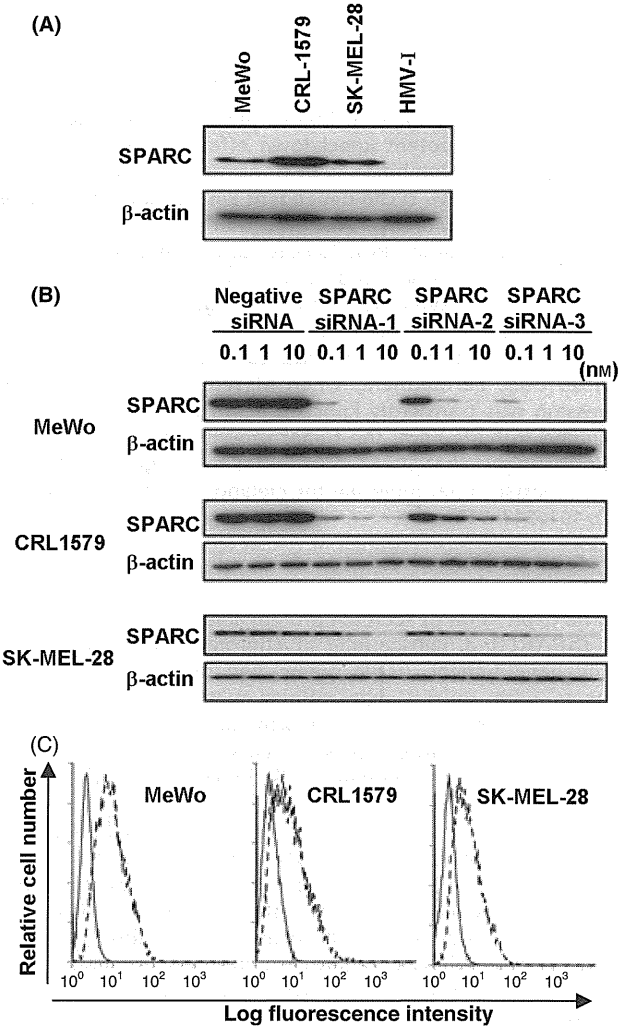

Fig. 1. Secreted protein acidic and rich in cysteine (SPARC) siRNA inhibits SPARC expression in melanoma cell lines. (A) The expression of SPARC protein in MeWo, CRL1579, SK-MEL-28, and HMV-I cells was analyzed using Western blot. β-actin was used as an internal control. (B) Knockdown efficiency of three SPARC siRNAs. At 48 h post-transfection with indicated concentrations, the expression of SPARC protein in MeWo, CRL1579, and SK-MEL-28 cells was analyzed using Western blot. β-actin was used as an internal control. (C) Transfection efficiency of siRNA on melanoma cells was assessed by flow cytometry at 24 h post-transfection with 50 nM FITC-conjugated negative control siRNA. The plot shows the relative cell number of melanoma cells (*y* axis) and the log fluorescence intensity (*x* axis).

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effect. Knockdown efficiency of SPARC protein was the highest in MeWo cells and the lowest in the SK-MEL-28 cells. These results suggest that these SPARC siRNAs successfully exert a silencing effect for SPARC expression. Transfection efficiency of siRNA was the highest in MeWo cells and the lowest in SK-MEL-28 cells (41.05%, 32.3%, and 27.97% at >10 of log fluorescence intensity in MeWo, CRL1579, and SK-MEL-28 cells, respectively) (Fig. 1C). This result was similar to knockdown efficiency among the melanoma cell lines.

Silencing of SPARC inhibits the growth of melanoma cells. We examined the effect of SPARC siRNA on the growth of melanoma cell lines. To check for non-specific side-effects of three siRNAs, we transfected SPARC siRNAs into SPARC non-producing cell line, HMV-I (Fig. 2A). As SPARC siRNA-2 showed a growth inhibition in HMV-I cells (data not shown), this siRNA was excluded from subsequent studies. SPARC siRNA-1 and -3 at 10 nM had no effect on cell growth. Therefore, we judged this concentration of SPARC siRNA to be reasonable in the experiments. SPARC siRNA-3 showed a marked growth inhibitory effect compared to SPARC siRNA-1 in MeWo and CRL1579 cells. The inhibition of the proliferation in MeWo cells was stronger than in CRL1579 cells. Silencing of SPARC hardly affected the growth of SK-MEL-28 cells. SPARC siRNA-3 inhibited the growth of MeWo cells in a dose-dependent manner, but did not significantly inhibit the growth of CRL1579 cells at 1 nM (Fig. 2B). These data indicate that silencing of SPARC can inhibit the growth of melanoma cell lines *in vitro*. Furthermore, we investigated whether the growth inhibition by silencing of SPARC in MeWo cells could be canceled by the addition of exogenous SPARC (Fig. 3A). As a source of exogenous SPARC, we prepared the conditioned media from MeWo cells transfected with negative control siRNA, or SPARC siRNA-3 (Fig. 3B). Western blot analysis revealed that the conditioned media from MeWo cells treated with negative control siRNA contained a substantial amount of SPARC protein. In contrast, SPARC protein in conditioned media from SPARC siRNA-3-treated MeWo cells was negligible. At 24 h post-transfection, conditioned media were swapped, and SPARC-silenced MeWo cells were cultured under both conditioned media. The growth of SPARC-silenced MeWo cells was not significantly different between SPARC-containing and SPARC-free conditioned media. These results indicate that intracellular SPARC, but not extracellular, is involved in the growth of melanoma cells.

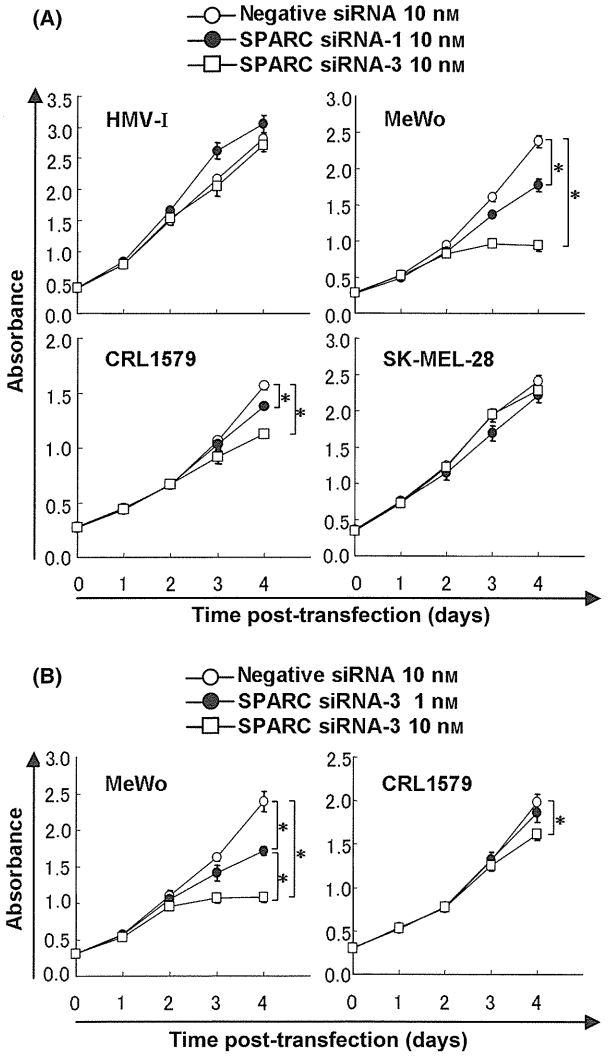

Fig. 2. Effects of secreted protein acidic and rich in cysteine (SPARC) knockdown on cell growth in melanoma cell lines. (A) HMV-I, MeWo, CRL1579, and SK-MEL-28 cells were transfected with SPARC siRNA-1, siRNA-3, or negative control siRNA at 10 nM. (B) MeWo and CRL1579 cells were transfected with SPARC siRNA-3 at the indicated concentrations. Cell growth was measured daily using WST-8 assay. The data at day 4 were statistically analyzed by one-way ANOVA followed by Dunnett's multiple comparison test (A) or Tukey's multiple comparison test (B). Error bars indicate SD. $^{*}P < 0.05$.

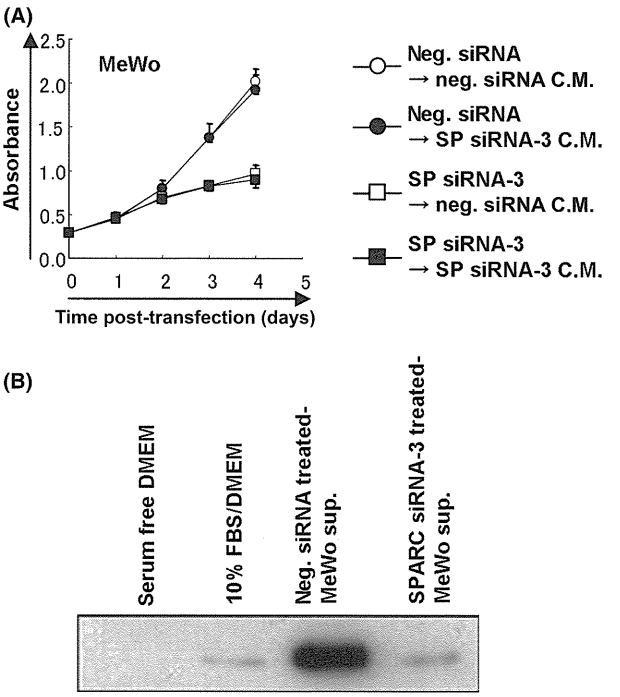

Fig. 3. Intracellular secreted protein acidic and rich in cysteine (SPARC), but not extracellular, is involved in the growth of melanoma cells. (A) Effect of exogenous SPARC on SPARC-silenced melanoma cells. MeWo cells were transfected with SPARC (SP) siRNA-3 (squares) or negative control (neg.) siRNA (circles) at 10 nM. After 24 h, cell culture media were swapped for MeWo cells treated with SP siRNA-3 (closed squares and circles) or neg. siRNA (open squares and circles). Cell growth was measured by using WST-8 assay. The data were statistically analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Error bars indicate SD. C.M., conditioned media. (B) The preparation of siRNA-treated MeWo cell conditioned media. The amount of SPARC protein in conditioned media was analyzed using Western blot. sup., supernatants.

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Inhibition of SPARC expression induces cell cycle arrest in melanoma cells. We examined the effects of SPARC siRNA on cell cycle progression. Silencing of SPARC in MeWo cells increased G_1 and decreased S phase populations at 72 h post-transfection with SPARC siRNA-1 or -3 (Fig. 4A). These results indicate that silencing of SPARC induces G_1 arrest. Similarly, the induction of G_1 arrest was observed in SPARC-silenced CRL1579 cells. However, no change was observed in SPARC-silenced SK-MEL-28 cells. To confirm G_1 arrest induced by SPARC siRNA, p21 and p53 protein expressions were investigated (Fig. 4B). When SPARC siRNA induced G_1 arrest in MeWo and CRL1579 cells, notable p21 induction was observed. The base level of p21 protein was very low in SK-MEL-28 cells. The accumulation of p21 protein was not observed in SPARC-silenced SK-MEL-28 cells. In SPARC-silenced MeWo cells, the level of p53 protein was increased compared with negative control siRNA-treated cells. However, it was not observed in SPARC-silenced CRL1579 or SK-MEL-28 cells. These results indicate that there is no correlation between p21 and p53 induction in CRL1579 and SK-MEL-28 cells. Taken together, these results indicate that the downregulation of SPARC induces growth inhibition with G_1 arrest and p53-dependent or -independent p21 accumulation in some melanoma cells.

Inhibition of SPARC expression enhances apoptosis in melanoma cells. We next tested whether silencing of SPARC induced cell death in melanoma cell lines. The treatment of

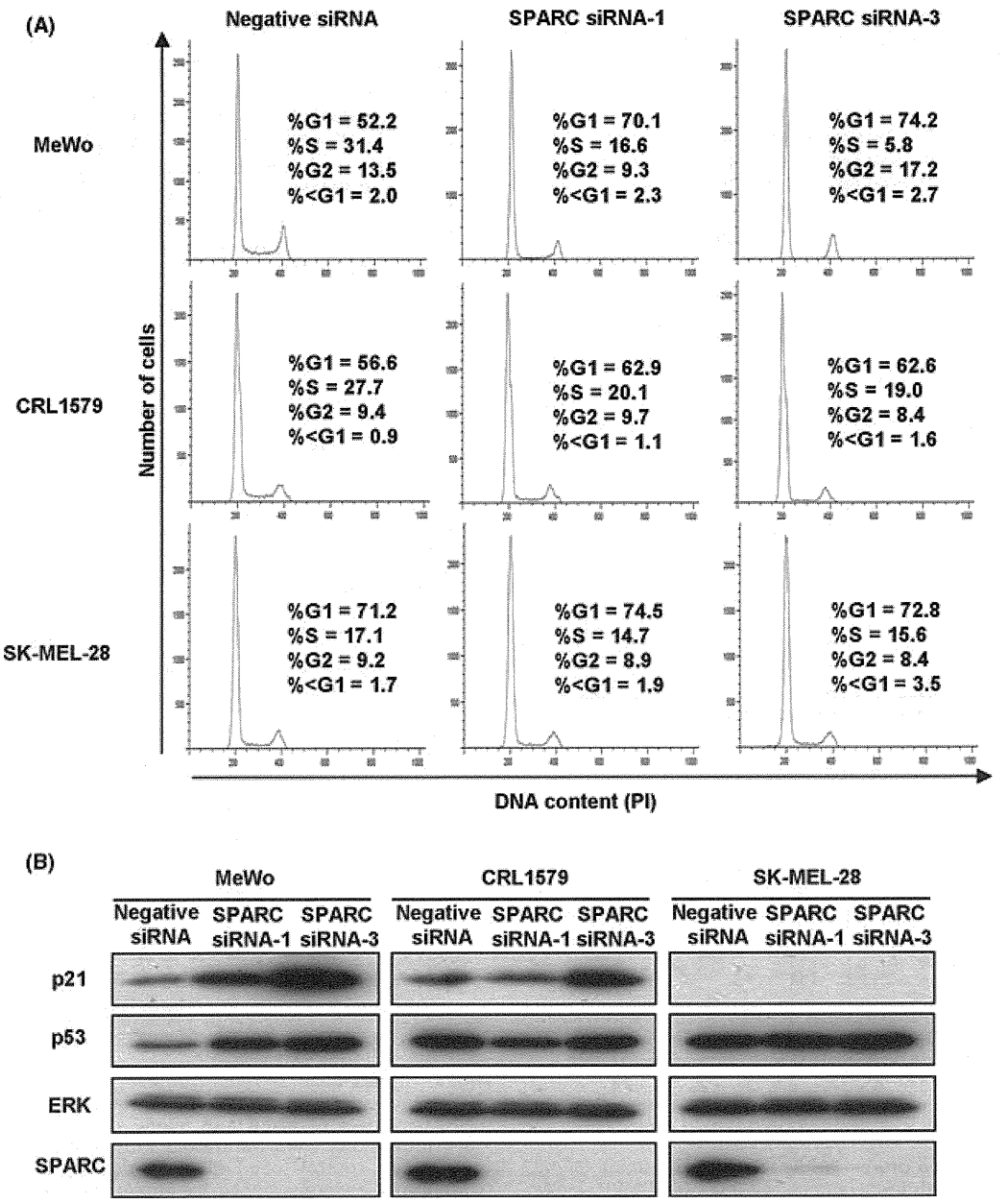

Fig. 4. Silencing of secreted protein acidic and rich in cysteine (SPARC) induces cell cycle arrest at G_1 phase in MeWo and CRL1579 melanoma cells. (A) Cell cycle distribution of melanoma cell lines transfected with SPARC siRNA. MeWo, CRL1579, and SK-MEL-28 cells were transfected with 10 nM SPARC siRNA-1, siRNA-3, or negative control siRNA. At 72 h post-transfection, DNA content was measured using propidium iodide (PI) staining on flow cytometry. The percentage of cells in each phase of the cell cycle is shown in each panel. (B) The expression of p21 and p53 proteins in MeWo, CRL1579, and SK-MEL-28 cells transfected with 10 nM SPARC siRNA-1, siRNA-3, or negative control siRNA. At 72 h post-transfection, total protein was analyzed using Western blot. ERK was used as an internal control.

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MeWo and CRL1579 cells with SPARC siRNA-3 increased (more than two-fold) early apoptotic cells as well as late apoptotic cells, compared with negative control siRNA treatment (Fig. 5). In SK-MEL-28 cells, the increase of apoptotic cells was not observed. Similarly, no difference was observed in the ratio of apoptosis between negative control siRNA and SPARC siRNA-3-treated HMV-I cells. These findings suggest that SPARC is involved in apoptosis to maintain cellular survival in some melanoma cells.

Silencing of SPARC inhibits growth of melanoma cells *in vivo*. We attempted to examine the effect of silencing of SPARC on tumor growth *in vivo* with a xenograft model. To assess the persistence of SPARC siRNA-mediated silencing, the kinetics of the downregulation of SPARC protein in MeWo cells *in vitro* was shown using Western blot analysis (Fig. 6A). The duration of the downregulation by SPARC siRNA was 6 days *in vitro*. At 8 days post-transfection, SPARC expression increased slightly. On the basis of these findings, we examined whether silencing of SPARC inhibited tumor growth *in vivo* using a xenograft model. A similar number of MeWo cells, transfected with SPARC siRNA-3 or negative control siRNA, were injected into both flanks of four nude mice. When tumors were palpable, their size was determined until 8 days post-implantation. As seen in Figure 6(B,C), the growth of tumors transfected with SPARC siRNA-3 was significantly suppressed compared with tumors transfected with negative control siRNA. At 8 days post-implantation, the growth inhibition of mice implanted with 1×10^6 or 3×10^5 cells transfected with SPARC siRNA-3 was 49% and 48%, respectively, as compared with negative control siRNA transfected cells ($P < 0.05$). These results indicate that downregulation of SPARC suppresses tumor growth *in vivo*.

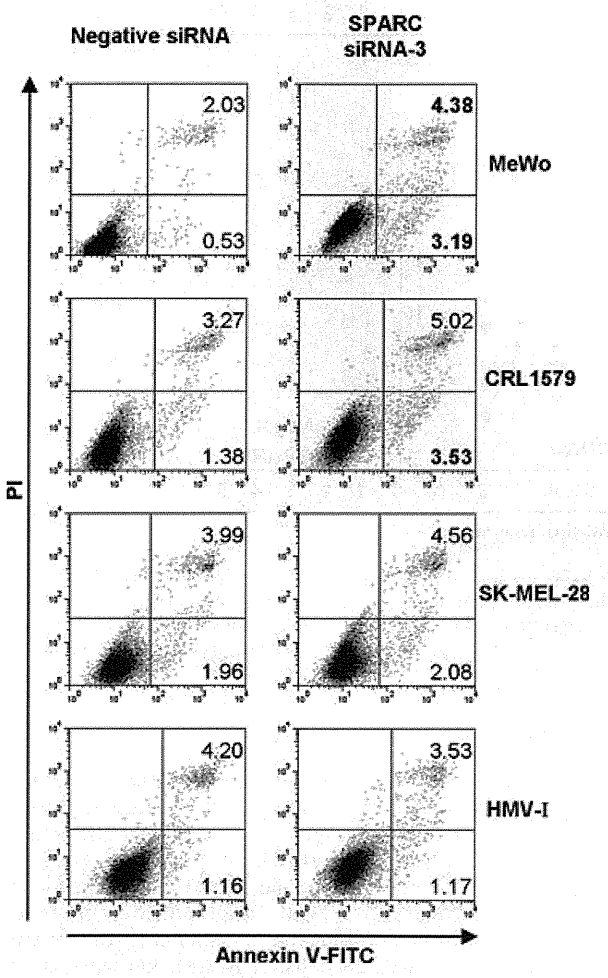

Fig. 5. Silencing of secreted protein acidic and rich in cysteine (SPARC) results in induction of early apoptosis in melanoma cell lines. For flow cytometric analysis, cells were harvested at 96 h after transfection with 10 nM SPARC siRNA-3 or negative control siRNA, then stained with annexin V–FITC and propidium iodide (PI). The percentages of annexin V^+/PI^- (early apoptotic) and annexin V^+/PI^+ (late apoptotic) cells is shown in each panel. Values in bold indicate more than a two-fold increase in apoptotic cells using SPARC siRNA-3, compared with negative control siRNA.

Discussion

The underlying mechanisms of growth regulation by SPARC in tumor cells are complicated. We focused on cell growth, and showed that SPARC produced from melanoma cells functionally linked to their own growth in this study. The most significant finding was that the downregulation of SPARC expression induced growth inhibition with G_1 arrest. This growth inhibitory effect by silencing of SPARC was maintained in an *in vivo* xenograft model.

We showed that there was a correlation with the level of p21 accumulation and the growth inhibition by silencing of SPARC. To examine whether induced p21 was involved in growth arrest by the silencing of SPARC, p21 was knocked down in MeWo cells using three siRNAs targeted to p21. The transfection with p21 siRNAs resulted in the reduction of the level of p21 protein accumulated using SPARC siRNA-3 (data not shown). However, the induction of p21 protein did not contribute to cell cycle arrest in our model, because growth inhibition by silencing of SPARC was maintained in cells even after knockdown of the p21 protein level (data not shown). These results suggest that p21 is not a major player in mediating the growth inhibition by silencing of SPARC. It is well known that G_1 arrest is regulated by Ink4 and Cip/Kip family proteins.[26] We have not yet examined the correlation between other Ink4 or Cip/Kip family proteins and G_1 arrest caused by silencing of SPARC. To better understand the mechanism of G_1 arrest induction, further investigations are needed to examine the expression of these proteins in SPARC-silenced cells.

It has been described that p21 is a transcriptional target of p53.[27] The expression of p21 was positively correlated with the expression of p53 in SPARC siRNA transfected MeWo cells, whereas p53 expression was not changed in SPARC siRNA transfected CRL1579 or SK-MEL-28 cells. Therefore, we speculate that p53-dependent or -independent p21 induction occurred in the melanoma cells treated with SPARC siRNA. The mechanisms for p53 induction in SPARC-silenced MeWo cells are unclear. Many forms of stress have now been shown to activate p53.[28,29] Although not examined in this study, it is possible that the loss of the protective effect of SPARC against some stress might affect p53 induction. Weaver *et al.* reported that SPARC protects lens epithelial cells from cell death induced by exposure to intracellular stressor, tunicamycin.[30] In addition, it has been reported that SPARC promotes glioma cell survival through Akt activation through integrin signaling under serum-free conditions.[20] These reports strongly suggest that SPARC plays a role as an antistress factor.

How does SPARC act? We showed that exogenous SPARC in culture conditioned media could not cancel the growth inhibition of MeWo cells with SPARC siRNA treatment. Thus, extracellular SPARC released from melanoma cells had no effect on cell proliferation in this system. We suggest that intracellular SPARC, but not secreted extracellular SPARC, contribute to cell growth or survival advantages. Martinek *et al.* have proposed intracellular SPARC functions as collagen-specific molecular

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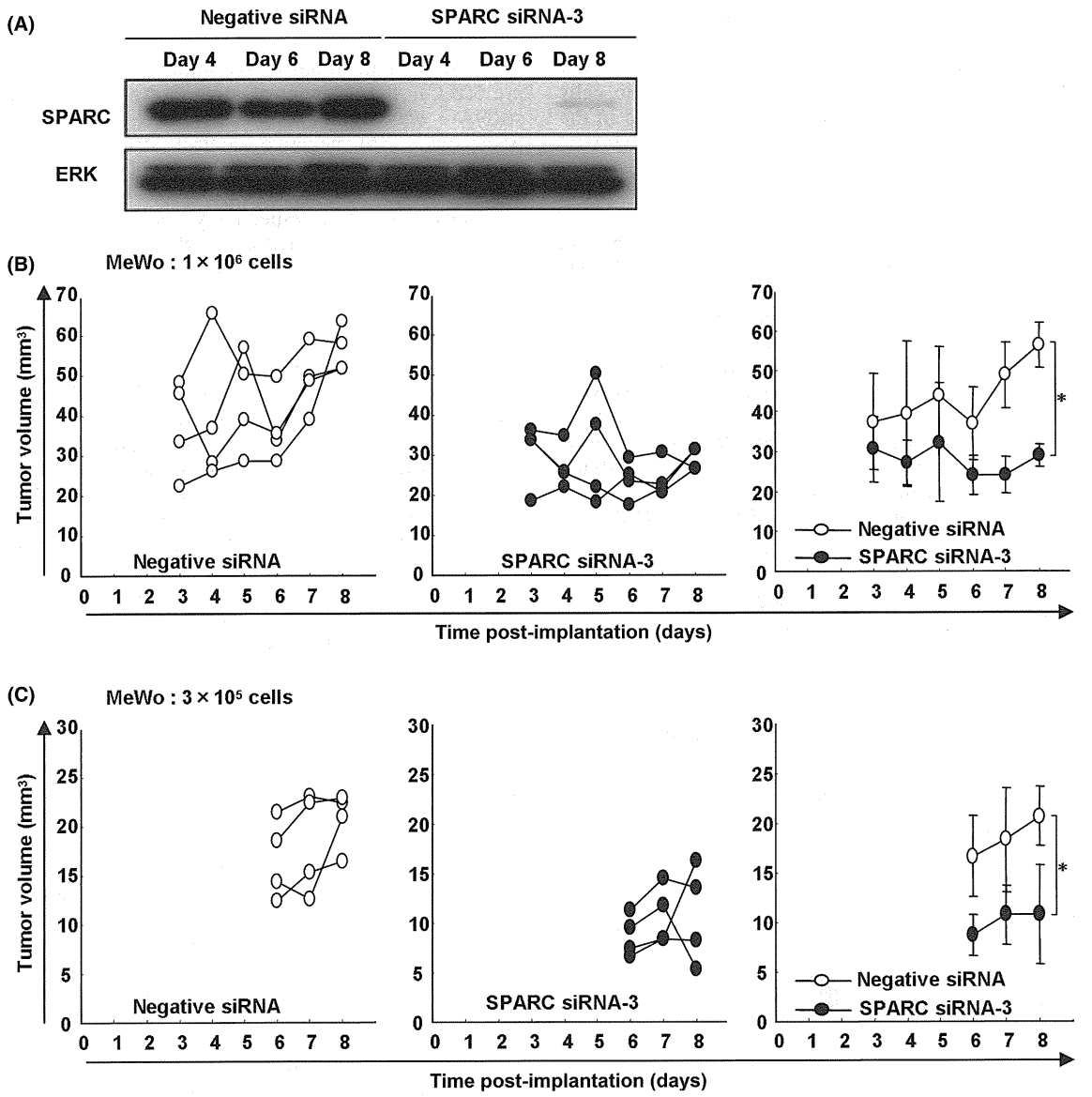

Fig. 6. Effect of secreted protein acidic and rich in cysteine (SPARC) knockdown on melanoma cell growth *in vivo*. (A) The duration of the knockdown effect of SPARC siRNA *in vitro*. At 4, 6, and 8 days post-transfection, SPARC protein was analyzed using Western blot. ERK was used as an internal control. MeWo cells were transfected with SPARC siRNA-3 or negative control siRNA in culture dishes. Twenty-four hours later, 1×10^6 (B) or 3×10^5 (C) tumor cells were s.c. implanted into both flanks of four nude mice. Individual tumor growth was measured with a caliper every day until 8 days post-implantation. Statistical differences were determined by Mann–Whitney *U*-test (B) or Student's *t*-test (C). The mean tumor volumes ± SD. $^*P < 0.05$ compared to negative control siRNA treatment at day 8.

chaperone, prior to their export from the endoplasmic reticulum.[31] Further study is needed to elucidate the roles of intracellular SPARC.

In our *in vivo* experiments, silencing of SPARC inhibited tumor growth, but did not lead to tumor rejection. This result might be due to the modest induction of apoptosis caused by silencing of SPARC. Other investigators showed that SPARC-silenced melanoma cells were abolished in *in vivo* xenograft models.[21] Their strategies for SPARC knockdown used SPARC downregulated stable cell lines. Their report suggests that it is important for the persistence of SPARC knockdown to abolish tumor cells. We need to further confirm the efficacy of the sequential administration of SPARC siRNA in the *in vivo* xenograft model.

Our results showed that there were differences in the degree of growth inhibition among SPARC siRNA-treated cell lines. Unlike MeWo and CRL1579 cells, the silencing of SPARC in SK-MEL-28 cells did not show growth inhibition. From our results, a reason for this might have been that the degree of knockdown of SPARC by siRNA in SK-MEL-28 cells was weaker than other cell lines. Second, the SPARC dependency on cell growth in SK-MEL-28 cells might have been less. We found that p53 was not induced by silencing of SPARC in CRL1579 and SK-MEL-28 cells. It is well known that p53 is important in the regulation of cell cycle checkpoints. Therefore, these results suggest that the regulation of cell cycle arrest by the checkpoint system in these cell lines might have been partial, compared with SPARC siRNA-treated MeWo cells. In addition, the antiproliferative effect of SPARC siRNA in CRL1579 cells was not clearly manifested, because the cell growth rate was lower in CRL1579 cells than in MeWo cells under ordinary culture conditions (data not shown). The differences of these

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inherent features of cell lines may have influenced the outcome of SPARC siRNA treatment.

Although the functional role of SPARC in cancer have been controversial, there are reports that it might play antitumorigenic roles in ovarian cancer.[32–34] It has been indicated that SPARC induces apoptosis in ovarian cancer cells.[32] In addition, SPARC normalizes the ovarian cancer microenvironment through vascular endothelial growth factor (VEGF) signaling modulation.[33] Furthermore, it has been reported that SPARC attenuates integrin-mediated signaling and Akt survival signaling in ovarian cancer cells.[34] The possible causes of these contradictory roles of SPARC in cancers might be the difference in tumor origin, properties of malignant cells, and tumor microenvironment. There is a need for further studies to clarify the roles of SPARC not only on cancer cells, but also on the interplay of tumor cells and tumor stroma.

In conclusion, we provided a new finding regarding the functional roles of SPARC on the regulation of cell proliferation in melanoma cell lines. The SPARC siRNAs we used exerted a potent knockdown effect. These siRNAs will be useful tools for therapeutic intervention by targeting SPARC. Further studies will elucidate the roles of SPARC on the growth of melanoma cells. All of this information contributes to a better understanding of SPARC as a potential therapeutic target for the treatment of melanoma.

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Glypican-3 expression predicts poor clinical outcome of patients with early-stage clear cell carcinoma of the ovary

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ABSTRACT

Background Glypican-3 (GPC3), a membrane-bound heparan sulphate proteoglycan, may play a role in promoting cancer cell growth and differentiation. Recent studies reported that GPC3 is overexpressed in clear cell carcinoma (CCC) of the ovary, and not other ovarian histotypes. However, in CCC patients, the relationship between the overexpression of GPC3 and prognosis has not yet been clarified.

Aim To evaluate GPC3 expression by immunohistochemistry in CCC.

Methods and Results In 52 CCC patients, GPC3 expression was observed in 40.4%. In cases of CCC, no correlations were identified between GPC3 expression and clinicopathological factors, such as age, FIGO stage, CA125 values, peritoneal cytology, ascitic fluid volume and mortality rate, except for the residual tumour size. GPC3 expression was associated with poor progression-free survival in stage I CCC patients. The numbers of Ki-67-stained cells in GPC3-positive areas were lower than those in GPC3-negative areas. GPC3 expression may be associated with a low proliferation rate in CCC cells. In the early stage of CCC, GPC3-expressing patients tended to be resistant to taxane-based treatment.

Conclusions Results suggest that the overexpression of GPC3 may be related to the low-level proliferation of tumours; it may be associated with resistance to taxane-based chemotherapy and a poor prognosis in CCC of the ovary.

INTRODUCTION

Epithelial ovarian carcinoma (EOC) is the leading cause of death from gynaecological malignancy. Since ovarian carcinoma frequently remains clinically silent, the majority of patients with this disease have advanced intraperitoneal metastatic disease at diagnosis.[1] In addition, various histological types and degrees of malignancy make it complicated to understand and analyse ovarian carcinoma, and the chemosensitivity and biological nature are different among these histological types.[2] Clear cell carcinoma (CCC) of the ovary was originally termed 'mesonephroma' by Schiller in 1939, as it was thought to originate from mesonephric structures and resemble renal carcinoma.[3] Since 1973, CCC has been recognised in the WHO classification of ovarian tumours as a distinct histological entity, and its clinical behaviour is also distinctly different from that of other epithelial ovarian cancers.[4] Several studies have shown that CCC patients exhibit a poor prognosis.[5–9] In the literature, the low-level response of CCC to conventional taxane-based chemotherapy is associated with a poor prognosis.[10 11] Several reports have shown that the lower-level proliferation of clear carcinoma cells may contribute to their resistance to chemotherapy.[12 13] However, the mechanism of resistance to chemotherapy in CCC has remained unclear.

Glypicans are a family of heparan sulphate proteoglycans that are linked to the exocytoplasmic surface of the plasma membrane through a glycosylphosphatidylinositol anchor. Six glypicans have been identified in mammals (GPC1–GPC6), and two in *Drosophila*.[14] The physiological function of glypicans is still not well understood. However, it was shown that the glypican-3 (GPC3)-encoding gene is mutated in patients with Simpson–Golabi–Behmel syndrome, an X-linked disorder characterised by prenatal and postnatal overgrowth and a varying range of dysmorphisms. GPC3 regulates cell growth either positively or negatively depending on the cell type. Genetic and functional studies showed that glypicans regulate the signalling activity of various morphogens, including Wnts, hedgehogs, bone morphogenic proteins and fibroblast growth factors.[15–19] Previous studies showed that GPC3 was overexpressed in Wilms' tumour, hepatocellular carcinoma and hepatoblastoma.[20 21] In ovarian carcinoma, GPC3 was overexpressed in yolk sac tumour and CCC, and not in other histotypes of EOC.[22–24] In a previous report, we demonstrated that GPC3 was associated with taxol resistance in CCC.[25] Thus, we hypothesised that GPC3 expression was associated with a poor prognosis in CCC patients. Maeda *et al* reported that GPC3 expression was significantly associated with a poor prognosis in stage III/IV CCC cases. However, the majority of clear cell adenocarcinomas are diagnosed at an early stage, and the relationship between GPC3 expression and the prognosis has remained unclear in early-stage CCC cases.

In the present study, we examined the immunohistochemical expression of GPC3 in CCC tissues to determine whether GPC3 expression is correlated with clinicopathological factors or the prognosis of CCC patients, especially in early-stage disease. We also investigated whether GPC3 was associated with CCC proliferation based on immunohistochemistry.

MATERIALS AND METHODS

Patients and tissue samples

Fifty-two human CCC tissue samples were obtained from patients who had undergone surgical

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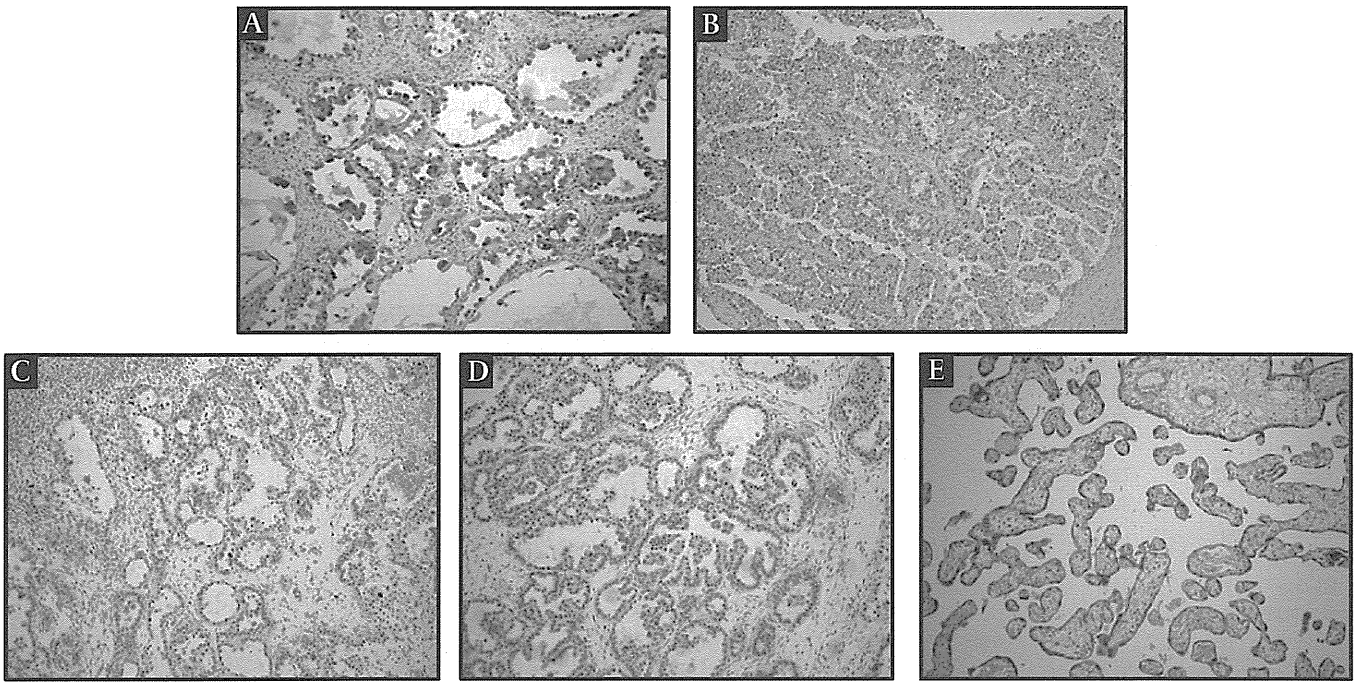

Figure 1 Immunohistochemical staining patterns for glypican-3 (GPC3) in clear cell carcinoma. (A) Strong positive expression of GPC3. (B) Moderate positive expression of GPC3. (C) Weak positive expression of GPC3. (D) Negative expression of GPC3. (E) Positive control for GPC3 (normal placenta).

treatment at Nagoya University Hospital between 1992 and 2006 after giving informed consent. The age of the patients ranged from 27 to 77 years, with a median of 52 years. None of these patients had undergone neoadjuvant chemotherapy before surgery.

All tissue samples were fixed in 10% formalin, embedded in paraffin and routinely stained with H&E for histological examination. All patients received postoperative chemotherapy with platinum plus cyclophosphamide and doxorubicin (before 1997) or platinum plus paclitaxel (after 1997). Tumour recurrence/progression was defined based on the clinical, radiological or histological diagnosis. Chemoresistance was defined as the appearance of a new lesion or a greater than 25% increase in tumour size within 6 months after finishing chemotherapy.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections were cut at a thickness of 4 μm. For heat-induced epitope retrieval, deparaffinised sections in 0.01 M citrate buffer (Target Retrieval Solution, pH 6.1, Dako, Glostrup, Denmark) were treated three times at 90°C for 5 min using a microwave oven. Immunohistochemical staining was performed using the avidin-biotin immunoperoxidase technique (Histofine SAB-PO kit, Nichirei, Tokyo, Japan). Endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide in methanol for 15 min, and non-specific immunoglobulin binding was blocked by incubation with 10% normal goat serum for 10 min. The sections were incubated at 4°C for 12 h with primary antibody against human GPC3 (1:200, clone IG12; BioMosaics, Burlington, Vermont, USA) and Ki-67 (1:200, clone MIB-1, Dako). The sections were rinsed and incubated for 30 min with biotinylated secondary antibody. After washing, the sections were incubated for 30 min with horseradish peroxidase-conjugated streptavidin and finally treated with 3-amino-9-ethylcarbazole in 0.01% hydrogen peroxide for 10 min. The slides were counterstained with Meyer's haematoxylin. The immunostaining intensity of GPC3 was scored semiquantitatively based on the per cent positivity of stained cells employing a 4-tiered scale as follows: for the evaluation of GPC3 expression,

Table 1 Immunohistochemical staining in clear cell carcinoma patients

Strongly positive	5 (9.6%)
Moderately positive	9 (17.3%)
Weakly positive	7 (13.5%)
Negative	31 (59.6%)

Table 2 Relationship between the expression of glypican-3 (GPC3) and clinicopathological parameters of clear cell carcinoma

		GPC3		
	Number	Negative	Positive	p Value
Total	52	31	21	
Age, years				
≤50	19	13	6	0.33
>50	33	18	15	
FIGO stage				
I	32	17	15	0.22
II–IV	20	14	6	
Residual tumour				
<1 cm	45	24	21	0.02
≥1 cm	7	7	0	
CA125, U/ml				
<250	37	20	17	0.20
≥250	15	11	4	
Peritoneal cytology				
Negative	30	19	11	0.52
Positive	22	12	10	
Ascitic fluid volume, ml				
<100	33	17	16	0.11
≥100	19	14	5	

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Table 3 Univariate analyses of several clinicopathological parameters in relation to the survival of patients with clear cell carcinoma

		Progression-free survival		Overall survival	
	Number	5-year survival, %	p Value	5-year survival, %	p Value
Total	52				
Age, years					
≤50	19	51.5	0.43	69.2	0.74
>50	33	69.7		66.7	
FIGO stage					
I	32	74.5	0.02	80.4	0.007
II–IV	20	45.0		49.1	
Residual tumour					
<1 cm	45	68.5	0.002	74.7	0.001
≥1 cm	7	28.6		28.6	
CA125, U/ml					
<250	37	72.0	0.07	77.0	0.04
≥250	15	45.7		51.9	
Peritoneal cytology					
Negative	30	81.8	0.009	86.1	0.003
Positive	22	49.0		54.9	
Ascitic fluid volume, ml					
<100	33	75.5	0.003	81.2	0.003
≥100	19	41.8		46.7	
Glypican-3 expression					
Negative	31	61.1	0.63	63.8	0.52
Positive	21	66.5		75.4	

the staining intensity was scored as 0 (negative), 1 (weak), 2 (medium) or 3 (strong). The extent of staining was scored as 0 (0%), 1 (1–10%), 2 (11–50%) or 3 (≥51%) according to the percentage of the positive staining areas in relation to the total cancer areas. The sum of the intensity and extent scores was used as the final staining score (0–6) for GPC3. Tumours with a final staining score of more than 3 were considered to show positive expression. The scoring procedure was carried out twice by two independent observers (each blinded to the other's score) without any knowledge of the clinical parameters or other prognostic factors. The concordance rate was over 95% between the observers.

Statistical analysis

The χ^2 test was also used to analyse the distribution of GPC3-positive cases, according to clinicopathological parameters. Survival analyses were conducted according to the life tables and Kaplan–Meier methods. Comparison of the survival between groups was performed with the log-rank test. Stat View software V.5.0 (SAS Institution, Cary, North Carolina, USA) was used for all statistical analyses, and $p<0.05$ was considered significant.

RESULTS

Immunohistochemical expression of GPC3 in EOC tissues

As figure 1 shows, the immunoreactivity of GPC3 was detected at various levels. There was little immunoreactivity of GPC3 in the tumour stroma. Among the 52 CCC specimens examined in this study, GPC3 was detected in 21 cases (40.4%) (table 1). GPC3 immunoreactivity, when categorised into negative versus positive expression, was not associated with the age, FIGO stage CA125 value, ascitic fluid volume or peritoneal cytology among the clinicopathological parameters tested (table 2). There were significant correlations between GPC3 expression and the residual tumour size. Residual tumour sizes were all within 1 cm in GPC3-positive cases; in seven of 24 patients, the residual tumour size was more than 2 cm in GPC3-negative cases.

Correlation of GPC3 expression with survival of CCC patients

The median overall survival (OS) of CCC patients was 54.4 months (range 4.3–199.4). In univariate analyses, FIGO stage, residual tumour presence after primary cytoreductive surgery, positive peritoneal cytology and ascitic fluid volume were significant predictors of both a poor OS and poor progression-free survival (PFS); the CA125 value was the only significant OS predictor (table 3). The 5-year OS rates of patients with a negative (n=31) and positive (n=21) expression of GPC3 were 61.1% and 66.5%, respectively (table 3). No significant association was observed between GPC3 and survival (p=0.52). However, in stage I cases, the PFS of GPC3-positive was poorer than that of GPC3-negative CCC patients, although this was not significant (p=0.05; figure 2A), and the OS of GPC3-positive tended to be poorer than that of GPC3-negative CCC patients (p=0.11; figure 2B).

Correlation of GPC3 expression with cell proliferation

On immunohistochemical analysis of Ki-67 in GPC3-expressing patients, Ki-67-stained cells in GPC3-positive areas were markedly less frequent than those in GPC3-negative areas (figure 3). This suggested that GPC3 was associated with low-level proliferation in CCC of the ovary.

Clinical features in taxane-resistant patients

Thirty-six of 52 CCC patients received taxane-based chemotherapy. Twelve patients were resistant to this chemotherapy. Table 4 summarises the clinical features of the 12 taxane-based chemotherapy-resistant patients. Three of them (27.3%) were positive for GPC3 expression. The FIGO stages of GPC3-expressing patients were all stage I. In stage I patients, 19 received taxane-based chemotherapy. There were ten GPC3-positive and nine GPC3-negative patients. The chemoresistance rate was 30% in GPC3-positive and 11% in GPC3-negative patients. This suggested that GPC3 expression tended to be

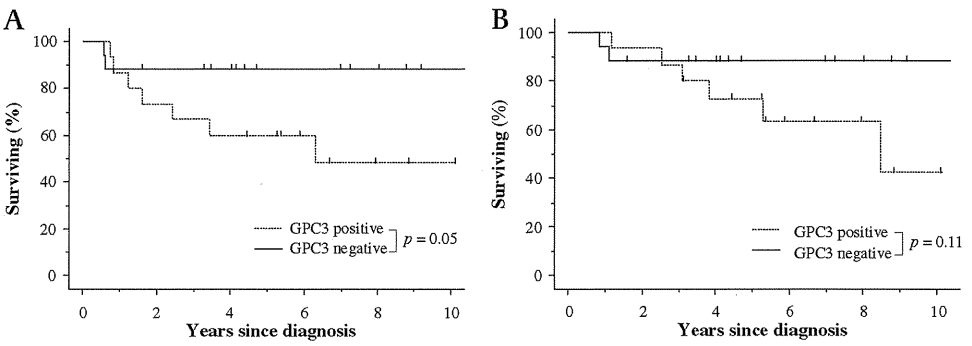

Figure 2 Progression-free survival (PFS) and overall survival (OS) curves drawn using the Kaplan–Meier method according to glypican-3 (GPC3) expression in stage I clear cell carcinoma patients. (A) PFS. (B) OS. Borderline significant differences in PFS (p=0.05).

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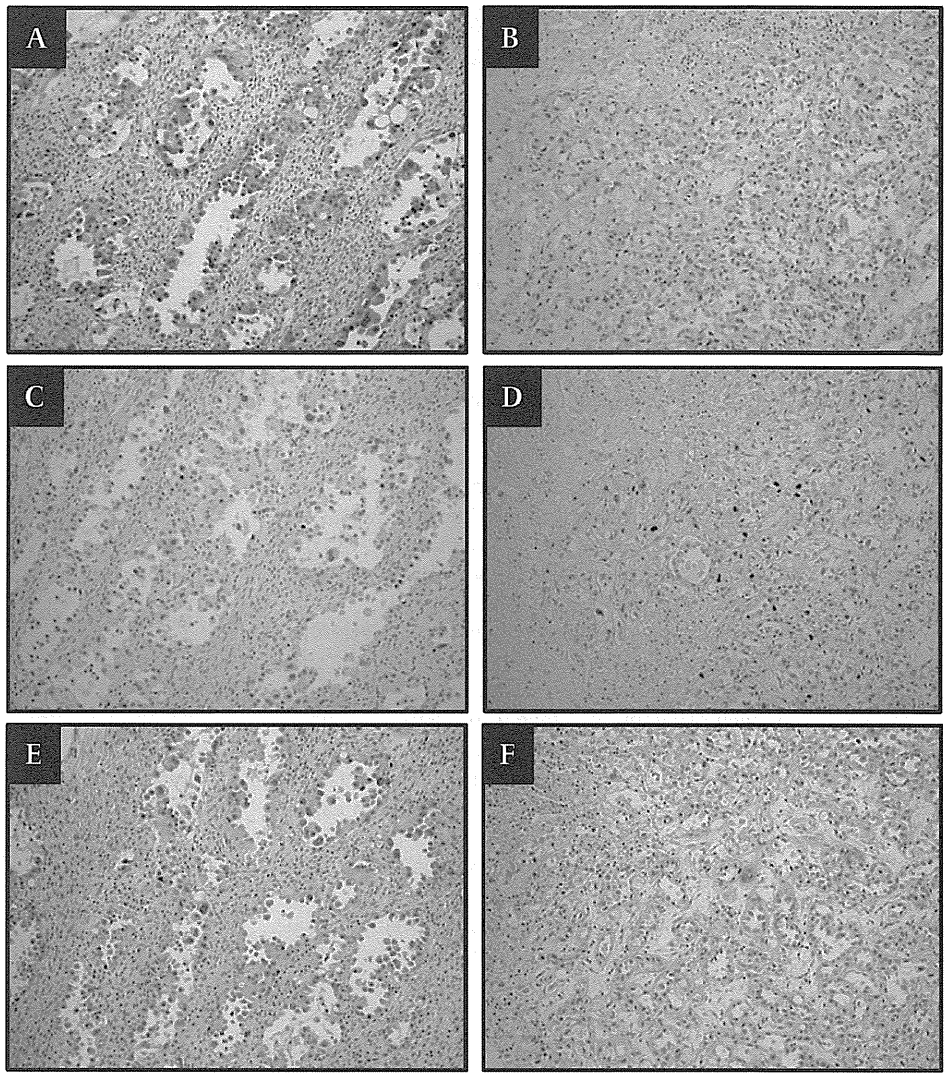

Figure 3 Immunohistochemical staining patterns for glypican-3 (GPC3) and Ki-67 in clear cell carcinoma; staining pattern of a tumour. (A) GPC3-positive. (B) GPC3-negative. (C, D) Ki-67. H&E staining was performed simultaneously for A and B. (E, F).

associated with resistance to taxane-based chemotherapy in early-stage CCC patients.

DISCUSSION

A previous study showed that GPC3 is exclusively overexpressed in CCC among ovarian adenocarcinomas. In this study, GPC3 was expressed in 40.4% of CCC. Maeda *et al* showed that GPC3 expression was significantly associated with poor overall survival in stage III/IV CCC patients. However, it remained unclear whether GPC3 was associated with prognosis in early-stage CCC patients. Thus, we evaluated whether GPC3 expression was correlated with prognosis in CCC patients. In this study, clinicopathological analysis of CCC indicated that GPC3 expression was associated with PFS in patients with stage I ($p=0.05$), although the number of stage I patients was small. However, several limitations of this investigation should be noted.

First and foremost are the limitations inherent to the reliability and reproducibility of immunohistochemical techniques.

Table 4 Clinical features of taxane-resistant patients

No.	Age, years	Stage	Initial operation	Residual tumour	Follow-up, months	Glypican-3 expression
1	40	1a	STH + BSO + OM + LN	–	38	Positive
2	53	1c	STH + BSO + OM + LN	–	14	Positive
3	40	1c	STH + BSO + OM + LN	–	14	Negative
4	50	1c	STH + RSO	–	31	Positive
5	38	2a	STH + BSO + OM + LN	–	24	Negative
6	53	2a	STH + BSO + OM + LN	–	18	Negative
7	38	2c	STH + BSO + OM + LN	–	41	Negative
8	65	3c	STH + BSO + OM	≤5 cm	8	Negative
9	58	3c	STH + BSO	≤1 cm	4	Negative
10	59	3c	STH + BSO + OM	≥5 cm	14	Negative
11	55	3c	Probe laparotomy	≥5 cm	9	Negative
12	54	4	Probe laparotomy	≥5 cm	9	Negative

STH, simple total abdominal hysterectomy; BSO, bilateral salpingo-oophorectomy; LN, retroperitoneal lymphadenectomy; OM, omentectomy.

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Take-home messages

- In ovarian carcinoma, glypican-3 (GPC3) was overexpressed in clear cell carcinoma (CCC), and not in other histotypes of epithelial ovarian carcinoma.
- GPC3 expression was associated with poor progression-free survival in stage I CCC patients.
- Using immunohistochemistry, it was shown that GPC3 expression may be associated with a low proliferation rate in CCC cells.

Immunohistochemistry is semiquantitative and highly dependent on a range of poorly controlled variables, including the antibody concentration, choice of antibody, variability in the interpretation and stratification criteria, and inconsistency in specimen handling and technical procedures. Another limitation of immunohistochemical staining is the variability in the commonly used visual scoring system. These scoring methods are subjective, and so subject to human variability.

In general, the prognosis of CCC patients is poor, largely due to a low response rate to taxane-based chemotherapy. In this study, GPC3 expression tended to be associated with resistance to taxane-based chemotherapy in stage I patients. We have already reported that GPC3 is associated with resistance to paclitaxel.[25] Our results showed the possible link between GPC3 expression and the paclitaxel-resistant nature of CCC in early-stage patients. Loss-of-function mutations of GPC3 are the cause of the X-linked Simpson–Golabi–Behmel syndrome (SGBS).[26] This disorder is characterised by developmental overgrowth.[27] GPC3-deficient mice also display developmental overgrowth along with several abnormalities found in SGBS patients.[28 29] Because the cell sizes are similar in GPC3 null mice and their normal littermates, it has been concluded that the increase in body size in the absence of GPC3 is the result of a higher cell proliferation rate. Consequently, it is reasonable to propose that the developmental overgrowth of SGBS patients and GPC3-deficient mice indicates that GPC3 acts as an inhibitor of cell proliferation in the embryo. Likewise, GPC3 is an inhibitor of cell proliferation and can induce apoptosis in certain types of tumour cell. CCC showed a significantly lower proliferation compared with other histotypes of EOC. This finding may explain the high incidence of stage I patients with CCC. It is known that rapidly proliferating cells are the most sensitive, whereas cells that slowly proliferate are generally less sensitive to cytotoxic agents. CCC patients showed a very low-level response to chemotherapy and a high incidence of progressive disease. Accordingly, chemoresistance may be an important factor in the poor prognosis of CCC patients. Low proliferation activity may contribute to chemoresistance and the poor prognosis in CCC. This study suggested that GPC3 was associated with low-level proliferation, making CCC resistant to taxane-based chemotherapy. Further functional experiments, including an in vitro study, are needed to elucidate the relationship between GPC3 and proliferation.

In conclusion, we found that GPC3 expression was associated with a poor prognosis in early-stage CCC patients. These results indicate that GPC3 is a reliable and promising prognostic indicator in early-stage CCC patients, and might become a novel molecular target in the treatment strategy for CCC.

Competing interests None.

Provenance and peer review Not commissioned; externally peer reviewed.

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Growth-suppressing function of glypican-3 (GPC3) via insulin like growth factor II (IGF-II) signaling pathway in ovarian clear cell carcinoma cells

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ABSTRACT

Objective. Ovarian clear cell carcinoma (CCC) is well known to be highly resistant to platinum-based chemotherapy. Glypican-3 (GPC3), a membrane-bound heparan sulfate proteoglycan, is overexpressed in only CCC of epithelial ovarian carcinoma subtypes. The purpose of this study was to identify the role of GPC3 in ovarian CCC.

Methods. To evaluate the function of GPC3 in ovarian CCC cells, we generated an ovarian cancer cell line, KOC7C cells stably transfected with plasmids encompassing shRNA targeting GPC3 (shGPC cells), and compared cell growth and the colony-forming ability to control shRNA-transfected cells (shCon cells).

Results. We showed that shGPC3 cells significantly increased cell growth and the colony-forming potential compared with shCon cells in 1% serum containing medium with 100 ng/ml IGF-II. Furthermore, these effects were significantly attenuated by pretreatment with 1 μM wortmannin (an inhibitor of PI3K/Akt).

Conclusions. We have demonstrated for the first time the presence of elevated levels of GPC3 protein associated with cell growth inhibition in CCC cells. Our data suggest that GPC3 has the potential to become a novel therapeutic target for ovarian CCC patients.

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Introduction

Ovarian cancer is the 5th leading cause of cancer death in women in the United States, with approximately 21,600 new cases and 15,500 deaths reported annually. In Japan, it is the eighth most common cause of cancer death, with approximately 7,700 new cases (2001) and 4,500 deaths (2007) reported yearly, and the incidence is increasing (Health, Labour and Welfare Ministry, Japan: Population Survey Report). More than 20% of all cases of ovarian cancer in Japan are classified as clear cell carcinoma (CCC) of the ovary, and, for unknown reasons, this percentage is markedly higher than in Europe and the United States. Compared with other epithelial ovarian carcinoma subtypes, ovarian clear cell carcinomas are associated with a poorer prognosis and a relatively increased resistance to platinum-based chemotherapy [1,2]. Thus, there is an urgent need to further our understanding of the pathogenesis of ovarian CCC, particularly with respect to the expression of proteins, which confer chemoresistance, for the development of a novel therapeutic strategy.

Glypican-3 (GPC3) is a 60-kDa cell-surface protein that belongs to the family of heparan sulfate proteoglycans (HSPGs), whose members are bound to the cell surface by a glycosylphosphatidylinositol (GPI) anchor [3]. HSPGs are expressed widely on both the surface of cells and within the extracellular matrix. GPC3 was first introduced as a possible tumor marker of HCC by observing significantly high levels of this protein in the serum of HCC patients, whereas it is undetectable in the serum of healthy donors and patients with benign liver diseases [4]. GPC3 stimulates the growth of HCC in vitro and in vivo by facilitating the interaction between Wnts and their signaling receptors [5]. In addition to HCC, the overexpression of GPC3 is also observed in Wilms tumor [6], malignant melanoma [7], ovarian cancer [8], and testicular germ cell tumor [9]. Mutations in the genes encoding both enzymes in the heparan sulfate biosynthetic pathway, as well as those encoding specific core proteins that bear heparan sulfate, have been shown to result in significant developmental abnormalities due to defects in growth factor signaling. Simpson–Golabi–Behmel syndrome, an X-linked condition characterized by pre- and postnatal overgrowth, whose features include numerous developmental abnormalities, tissue overgrowth, and an increased risk of embryonal malignancies, shows a GPC3 gene mutation [10]. GPC3 knockout mice exhibit several features of SGBS. Some of these dysmorphisms could be the result of deficient growth inhibition or apoptosis in certain cell types during development, suggesting that GPC3 plays a negative role in cell proliferation and apoptosis induction in some tissues. GPC3 has also been reported to bind insulin-like growth factor-II [11]. The insulin-like growth factor (IGF) axis is an important regulator of metabolic function, cellular development, and growth, and comprises two growth factors (IGF-I and IGF-II), IGF-

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binding proteins 1–6 and IGF-binding-protein-related proteins, and two principal cell membrane receptors (IGF-IR and IGF-IIR) that are homologous with the insulin receptor. Recent studies have implicated perturbations of the IGF axis in human malignancy, principally by the dysregulation of cellular growth, differentiation, and apoptosis, resulting in cellular transformation via paracrine and autocrine mechanisms [12,13].

In previous studies, it was hypothesized that GPC3 binds and sequesters or downregulates IGF-II [10,14], but Sung YK et al. showed that IGF-II signaling is not modulated by GPC3 [15], and Cheng et al. showed that GPC3 activates the IGF-II signaling pathway. In addition, this report showed that GPC3 is associated with IGF-II and IGF-IR through its proline-rich region, which might be important for protein–protein interaction, and GPC3 may regulate the phosphorylation of IGF-IR [16]. These results show that the role of GPC3 in the IGF-II-dependent pathway in cancer cells is still unclear.

In order to investigate the functions of GPC3 in ovarian CCC, we knocked down GPC3 in a GPC3-expressing ovarian CCC cell line (KOC7c). We demonstrated specific interactions both between GPC3 and IGF-II and between GPC3 and IGF-IR, and we also showed that GPC3 is involved in the Akt signaling pathway.

Materials and methods

Cell lines and treatment

The human clear cell adenocarcinoma cell line KOC7c was cultured in RPMI 1640 supplemented with 10% w/v fetal bovine serum (FBS), penicillin at 100 U/ml, and streptomycin at 100 μg/ml, (all from Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified atmosphere of 5% CO_2.

Roscovitine (Sigma, St Louis, Missouri, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma), and final concentrations of 10, 20, and 40 M were used to treat the cells and an appropriate amount of DMSO was used as a vehicle control. For combination treatment, cells were pretreated with different concentrations of wortmannin (Sigma) for 40 min followed by roscovitine treatment for 24 h.

Inhibition of GPC3 expression by short hairpin RNA

Short hairpin RNA (shRNA) of human *GPC3* was purchased from Qiagen-Xeragon (Germantown, MD, USA). The target site for *GPC3* shRNA was 5-CCAATGCCATGTTCAAGAATTCAAGAGATTCTTGAA-CATGGCATTGGTTTTT-3 and its antisense oligonucleotide. The oligonucleotides were annealed and inserted into an RNAi-Ready pSIREN-RetroQ-TetP vector (Takara Bio, Tokyo, Japan).

To establish stable cell lines, the vector plasmid was transfected into parental KOC7c cells using Lipofectamine 2000 reagent (Invitrogen, San Diego, CA, USA) and then selected with puromycin.

Proliferation assay

To study the effect of silencing GPC3 on KOC7c cell growth, 1×10^4 cells transfected with GPC3 shRNA (shGPC3) or control shRNA (shCon) cells were seeded in 35-mm dishes in RPMI 1640 supplemented with 1% FBS or with 1% FBS plus recombinant human IGF-II (100 ng/ml). Cells were harvested at 48-h intervals and counted in triplicate. Viable cells were counted at 48 h intervals for 14 days.

Colony formation assay

shGPC3 or shCon cells were plated at a density of 200 cells in 60-mm culture dishes (ASAHI GLASS CO, Chiba, Japan) in RPMI 1640 supplemented with 1% FBS or with 1% FBS plus recombinant human IGF-II (100 ng/ml) with or without pretreatment of wortmannin (1 μM) and PD98059 (20 μM) and stained with May–Grünwald Giemsa staining on day 14. The culture medium was changed every 2 days. Colony diameters larger than 500 μm were then counted as 1 positive colony. The number of colonies was counted per 2.25 cm^2, and we performed five individual experiments.

Western blotting

Proteins were resolved employing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel, and transferred onto an activated polyvinylidene difluoride (PVDF) membrane in cold transfer buffer (14.4 g of glycine, 3 g of Tris base, and 1 g of SDS dissolved in 1 L of 20% methanol) at 30 V overnight. The membrane was then blocked for 1 h with 5% non-fat milk dissolved in Tris-buffered saline (TBS) containing 0.1% Tween-20, and probed with DNA-PK antibody diluted in 1% blocking buffer overnight at 4 °C. After blocking, the membrane was incubated for 1 h with the relevant antihuman primary antibody at the recommended dilution, GPC3 (BioMosaics, Burlington, VT, USA), or IGF-IR (ABgent, San Diego, USA), and β-actin (Abcam, Cambridge, MA, USA). The membrane was then washed three times with Tween / PBS for 15 min, and then incubated with the appropriate secondary antibody for 1 h. After washing with Tween/PBS, the membrane was treated with ECL-Western blotting detecting reagent (Amersham Biosciences KK, Tokyo, Japan).

Immunohistochemistry

Sections (5 μm) from one representative block from each case were deparaffinized, rehydrated in a graded alcohol series, and subjected to heat-induced epitope retrieval in 0.1 mol/L citrate buffer at pH 6.0 in a microwave for 20 min. The slides were then incubated with a primary monoclonal antibody specific for GPC3 with a dilution of 1:100 for 1 h at room temperature. After incubation with rabbit anti-mouse secondary antibody, a subsequent reaction was performed with biotin-free horseradish peroxidase enzyme-labeled polymer of the EnVision plus detection system (Dako Corporation, Carpinteria, CA, USA). 3,3'-Diaminobenzidine was used as the chromogen (Dako), and the sections were counterstained with hematoxylin. Each case was examined for cytoplasmic and membranous staining. In addition, each histologic pattern was independently analyzed.

Results

GPC3 expression in ovarian cancer tissues

We first examined GPC3 expression in surgically resected ovarian carcinoma tissues (Fig. 1). GPC3 was expressed in tumor cells from 40% of CCC (12/30) to 100% of yolk sac tumor tissues (8/8); although other histotypes were not stained, GPC3 expression was observed in intracellular lesions as well as in the cell membrane, suggesting that cancer cells showed an increased production of GPC3.

Effect of silencing GPC3 using shRNA on growth of KOC7c cells

In order to study the function of GPC3 on long-term growth in vitro, we used a shRNA technique targeting GPC3. GPC3 shRNA (shGPC3 cells) and control shRNA (shCon cells) were used to transfect KOC7C cells which were strongly positive for GPC3 expression. The cells were further cultured in selective medium. After 8 weeks, the cell clones were selected and seeded for further expansion in selective medium for Western blot analysis. The protein level of shGPC3 cells was clearly decreased compared to that of shCon cells. In contrast to GPC3, IGF-IR and β-actin were not affected (Fig. 2A). There was no significant difference in cell proliferation between shGPC3 and shCon cells in 1% serum containing medium for 14 days (Fig. 2B). On the

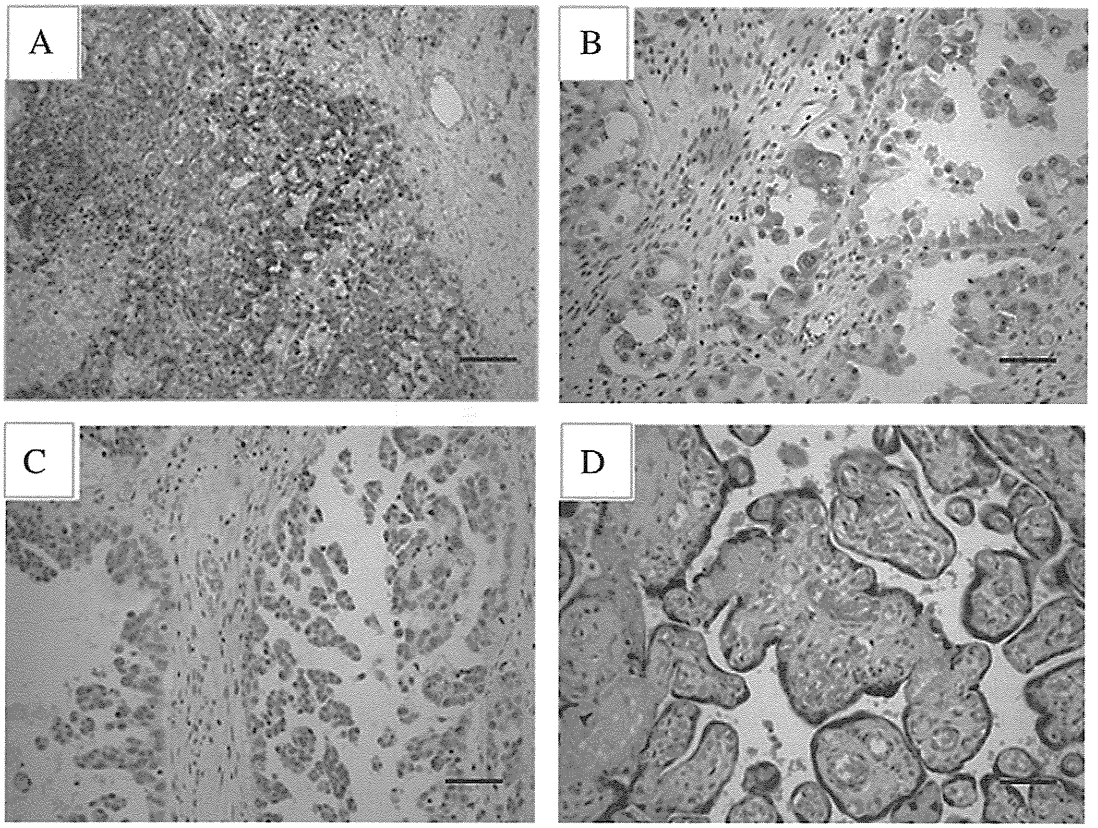

Fig. 1. Immunohistochemical analysis of GPC3 in malignant ovarian tumors. (A) Strongly positive case of ovarian yolk sac tumor. (B) Moderately positive case of ovarian CCC. (C) Negative case of ovarian CCC. (D) Positive control of placental tissue. Scale bar = 20 μm.

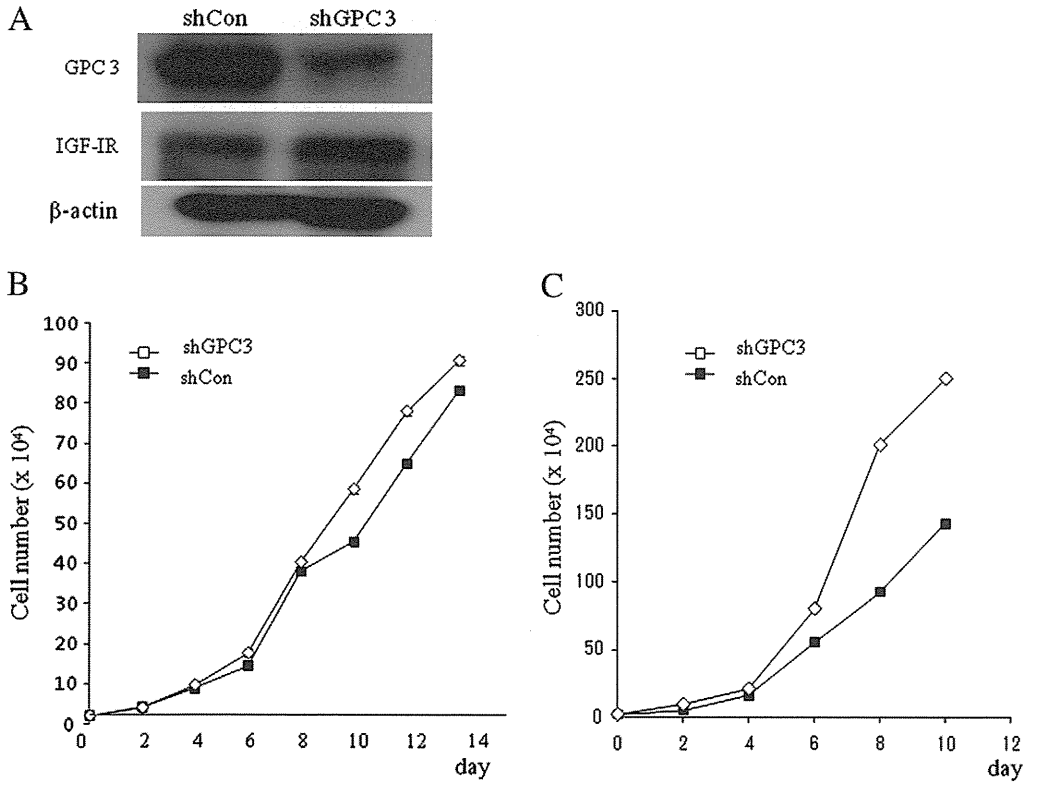

Fig. 2. (A) Expressions of GPC3 and IGF-IR in KOC7C (ovarian CCC) cells. Cells were transfected with GPC3 shRNA (shGPC3 cells) and control shRNA (shCon cells). (B) Cell proliferation assay with shGPC3 and shCon cells in 1% serum containing medium. (C) Cell proliferation assay with shGPC3 and shCon cells in 1% serum containing medium with 100 ng/ml IGF-II.

other hand, shGPC3 cells showed significantly increased cell growth compared with shCon cells in 1% serum containing medium with 100 ng/ml IGF-II at 12 days (Fig. 2C).

Effect of silencing GPC3 using shRNA on liquid colony formation potential of KOC7c cells

In order to document the ability of shGPC3 cells to form colonies, single-cell suspensions were plated at a density of 200 cells in 60-mm culture dishes in the limiting dilution experiment with 100 ng/ml IGF-II. As shown in Fig. 3A, after 10 days, colonies (i.e. cellular aggregates larger than 500 μm/2.25 cm^2) were formed much more efficiently by shGPC3 cells, which gave rise to a 4-fold larger number of colonies than that documented in shCon cells (*$p<0.001$) (Fig. 3B).

PI3K/Akt was involved in GPC3-mediated KOC7c liquid colony formation

To explore whether shGPC3-induced KOC7c cell colony formation with 100 ng/ml IGF-II was related to the PI3K/Akt pathways, shGPC3 and shCon cells were plated at a density of 1×10^3 cells and pre-incubated with 1 μM wortmannin (an inhibitor of PI3K/Akt) for 30 min. Following this, these cells were treated with 100 ng/ml IGF-II. These treatments were performed at 2-day intervals. As shown in Fig. 4, the inhibitor significantly attenuated the effect of IGF-II on shGPC3 cell colony formation. As shown in Fig. 4, the inhibitor did not decrease the effect of IGF-II on shCon cells. After 10 days, colony diameters larger than 500 μm/2.25 cm^2 were formed much more efficiently by shGPC3 cells, which gave rise to a 5-fold smaller number of colonies than that documented in shCon cells (**$p<0.001$). The results above suggested that the PI3K/Akt signaling pathway was involved in the GPC3-induced inhibition of colony formation with IGF-II in KOC7C cells. On the other hand, inhibition of the ERK pathway with PD98059 (20 μM) did not block the effect of IGF-II on shGPC3 cell colony formation (Fig. 5) (***$p<0.05$). Thus, these results showed that the ERK signal pathway was not involved in the GPC3-induced inhibition of colony formation with IGF-II in KOC7C cells.

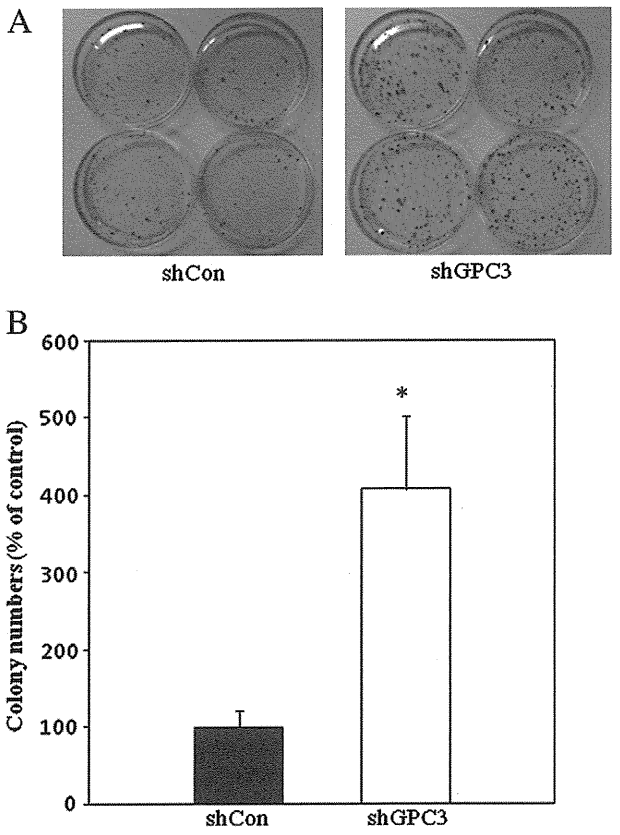

Fig. 3. Effect of GPC3 silencing using shRNA on the liquid colony formation potential of KOC7C cells. Cells were seeded in 35-mm plates and cultured in serum 1% medium containing IGF2 at 100 ng/ml. Colonies were stained with May–Giemsa and we counted those <1 mm. *$P<0.01$.

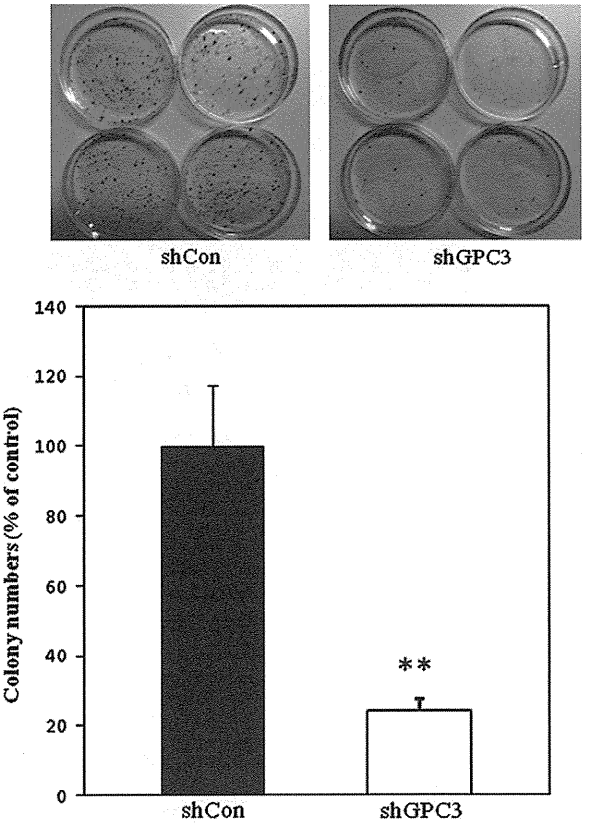

Fig. 4. Involvement of the PI3K/Akt pathway in GPC3-mediated KOC7C liquid colony formation. Cells were seeded in 35-mm plates and cultured in serum 1% medium with IGF2 (100 ng/ml) and wortmannin (1 μM). Colonies were stained with May–Giemsa, and we counted those <1 mm.

Discussion

Despite recent developments in carboplatin and paclitaxel combination chemotherapy, patients with CCC of the ovary, especially with advanced-stage or recurrent disease, show poor progression-free survival and overall survival rates when compared showing a patient with serous histology [17–20]. Therefore, to improve survival, new strategies are necessary to more effectively treat CCC.

In this study, we showed that the knockdown of GPC3 caused growth promotion in CCC cell lines in an IGF-II-dependent way. The IGF-signaling pathway plays an important role in ovarian cancer [21]. Overexpressions of IGF-II and IGF-IR have been reported in human cancer [22]. In this study, we investigated the expressions of IGF-IR in shGPC3 and shCon cells, and no significant difference was shown between these cells.

Moreover, in a previous study, it was shown that GPC3 interacted with both IGF-II and its receptor, IGF-IR, and the expression of GPC3 increased hepatocellular carcinoma (HCC) cell growth [23]. We consider this difference to reflect the differential activity of the pathway in different cells. Furthermore, a recent study showed that soluble GPC3 inhibits the growth of HCC cells in vitro and in vivo by simultaneously blocking several pro-tumorigenic growth factors [24]. We hypothesized that soluble GPC3 secreted by shCon cells inhibited IGF-II signaling pathway and result in inhibited cell growth. In addition, we found that wortmannin (an inhibitor of PI3K/Akt) selectively inhibited the colony-forming effect

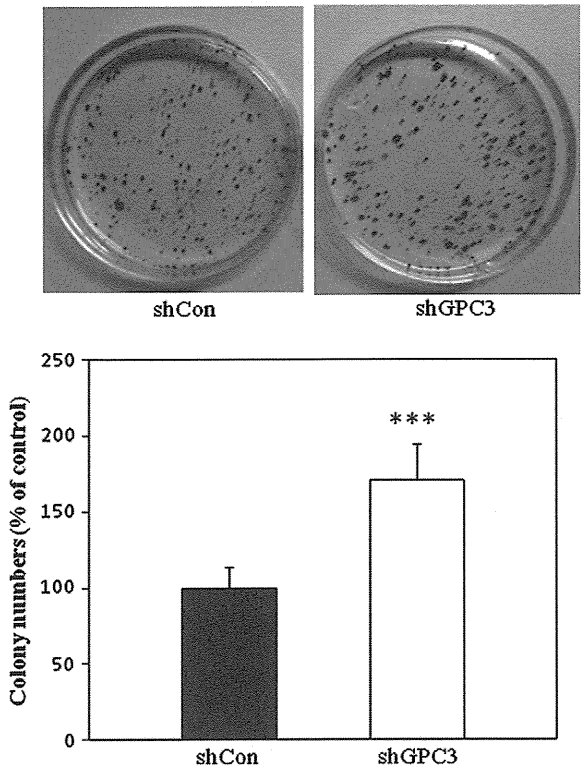

Fig. 5. Involvement of the ERK pathway in GPC3-mediated KOC7C liquid colony formation. Cells were seeded in 35-mm plates and cultured in serum 1% medium with IGF2 (100 ng/ml) and PD98059 (1 μM). Colonies were stained by May–Giemsa, and we counted those <1 mm.

of IGF-II in shGPC3 cells. These results suggested that IGF-II induced the PI3K/Akt pathway in shGPC3 cells but not in shCon cells.

In our previous study, we already reported that GPC3 was associated with resistance to Taxol [25]. On the other hand, loss-of-function mutations of GPC3 cause the X-linked Simpson–Golabi–Behmel syndrome (SGBS). This disorder is characterized by developmental overgrowth [10]. GPC3-deficient mice also display developmental overgrowth along with several abnormalities also found in SGBS patients [26]. Because cell sizes are similar in the GPC3 null mice and normal littermates, it has been concluded that the increase in body size in the absence of GPC3 is the result of a higher cell proliferation rate. Consequently, it is reasonable to propose that the developmental overgrowth of SGBS patients and GPC3-deficient mice indicates that GPC3 acts as an inhibitor of cell proliferation in the embryo. Likewise, GPC3 is an inhibitor of cell proliferation and can induce apoptosis in certain types of tumor cell. CCC showed significantly lower proliferation compared with other histotypes of epithelial ovarian cancer. This finding may explain the high incidence of stage I patients with CCC. It is known that rapidly proliferating cells are the most sensitive, whereas cells that slowly proliferate are generally less sensitive to cytotoxic agents. CCC patients showed a very low-level response to chemotherapy and a high incidence of progressive disease. Accordingly, chemoresistance may be an important factor in the poor prognosis of CCC patients, and the low proliferation activity may contribute to chemoresistance in CCC. Further investigation is required to clarify whether GPC3 is associated with low-level proliferation, leading to the resistance of CCC to chemotherapy.

In conclusion, our study demonstrated for the first time the elevated levels of GPC3 protein associated with cell growth inhibition in CCC cells. In the future, the detailed molecular mechanism by which GPC3 is involved in growth inhibition in CCC cells should be further investigated. Therefore, our study suggested that GPC3 has the potential to become a novel therapeutic target for ovarian CCC patients.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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