# Strong suppression of tumor growth by insulin-like growth factor-binding protein-related protein 1/tumor-derived cell adhesion factor/mac25

**Yuichiro Sato,[1,2] Zhaoxin Chen[1,2] and Kaoru Miyazaki[1,2,3]**

[1]Division of Cell Biology, Kihara Institute for Biological Research, Yokohama City University, 641-12 Maioka-cho, Totsuka-ku, Yokohama 244-0813; [2]Graduate School of Integrated Sciences, Yokohama City University, 641-12 Maioka-cho, Totsuka-ku, Yokohama 244-0813, Japan

(Received March 1, 2007/Revised March 23, 2007/Accepted March 26, 2007/Online publication April 30, 2007)

**Insulin-like growth factor binding protein-related protein 1 (IGFBP-rP1) has been shown to induce cellular senescence or apoptosis of breast and prostate cancer cell lines *in vitro*. To examine whether IGFBP-rP1 acts as a tumor-suppressive protein *in vivo*, we established two model systems. Expression of IGFBP-rP1 in the human bladder carcinoma cell line EJ-1 was blocked by RNA interference. Human colon cancer cell line DLD-1, which did not express endogenous IGFBP-rP1, was transfected with an IGFBP-rP1 expression vector. When injected intraperitoneally or subcutaneously into nude mice, the IGFBP-rP1-expressing EJ-1 and DLD-1 cell lines grew poorly, whereas the IGFBP-rP1 non-producers grew rapidly and produced large tumors. In monolayer culture the IGFBP-rP1 producers and non-producers grew similarly in each model, whereas in soft agar culture the former produced far less colonies than the latter. The IGFBP-rP1 producers had IGFBP-rP1 bound to the cell surface, and adhered more efficiently to fibronectin and laminin-5 than the respective non-producers. Expression of IGFBP-rP1 did not affect the efficiency of insulin signaling. These results demonstrate that IGFBP-rP1 strongly suppresses tumor growth by an insulin-independent or insulin-like growth factor-independent mechanism. Cell surface IGFBP-rP1 may reduce the anchorage-independent growth ability, leading to the marked loss of tumorigenicity. (*Cancer Sci* 2007; 98: 1055–1063)**

Insulin-like growth factor (IGF) binding protein (IGFBP)-related protein-1 (IGFBP-rP1) is a member of the IGFBP superfamily.[1,2] IGFBPs bind IGFs strongly but bind insulin weakly,[3–5] whereas IGFBP-rP1 binds both IGF and insulin with very low affinity.[5–7] The amino acid identity of IGFBP-rP1 compared with IGFBPs is as low as approximately 20%, which is far lower than that among the six members of the classical IGFBP family (~50%). The cDNA of IGFBP-rP1 was originally cloned as *mac25*, which was expressed in normal human leptomeningial cells but scarcely in meningiomas.[8] The protein product of IGFBP-rP1 was identified as a tumor-derived cell adhesion factor (TAF) from human bladder carcinoma cells[9] and as prostacyclin-stimulating factor from human fibroblasts.[7] The purified IGFBP-rP1 exerts a weak cell adhesion activity through heparan sulfate proteoglycans on the cell surface[10–12] and stimulates cell growth in culture medium containing insulin or IGFs.[6,13] Because this protein is highly accumulated in blood vessels of various human cancer tissues, it is also referred to as angiomodulin.[10] In colon cancers, the IGFBP-rP1 protein is detected in invading cancer cells.[14] The IGFBP-rP1 mRNA and protein are also expressed in a wide range of normal tissues, including the heart, spleen, ovary, small intestine and colon.[15] In IGFBP-rP1 knockout mice, significant changes are observed in ovaries, muscle tissues and liver even though they are viable.[2]

In contrast to the characteristics of IGFBP-rP1 described above, other studies have suggested that IGFBP-rP1 may function as a tumor suppressor protein in some types of tumors, especially mammary carcinomas and prostate carcinomas. IGFBP-rP1 expression is increased with cell senescence in normal human mammary epithelial cells, and it is slightly expressed or absent in mammary epithelial cell lines.[16] Hypermethylation of the CpG islands of the IGFBP-rP1 gene *mac25* is associated with its downregulation in hepatic carcinoma.[17] Introduction of the exogenous IGFBP-rP1 gene into mammary cancer cells significantly inhibits cell growth by a senescence-like mechanism *in vitro*.[18] In breast cancer patients, lower expression of IGFBP-rP1 in the cancers is correlated with worse prognosis compared with higher expression.[19] IGFBP-rP1 is found in normal human prostate tissues, but it is poorly or scarcely expressed in human prostate cancer tissues and cell lines.[20] Overexpression of an exogenous IGFBP-rP1 gene in prostate cancer cells inhibits cell growth under both anchorage-dependent and -independent culture conditions, inducing apoptosis.[21,22] Moreover, recombinant IGFBP-rP1 protein has been shown to inhibit the growth of some cancer cell lines.[23]

As shown above, there are some discrepancies in previous studies, especially regarding the growth-inhibitory or stimulatory activity and expression of IGFBP-rP1 in human cancer tissues. Furthermore, it remains unclear whether IGFBP-rP1 indeed suppresses tumor growth *in vivo*. To address these issues, we have established two models of human cancer cell lines by introducing an exogenous IGFBP-rP1 gene or using small interfering RNA (siRNA). Using these models, the functions of IGFBP-rP1 both *in vivo* and *in vitro* were investigated in this study.

## Materials and Methods

**Materials.** IGFBP-rP1 was purified from the conditioned medium of human bladder carcinoma cell line EJ-1, as described previously.[13] An anti-TAF/IGFBP-rP1 monoclonal antibody (#88) was obtained.[10] Human recombinant laminin-5 was prepared as previously reported.[24] Human fibronectin was purchased from Chemicon (Temecula, CA, USA). All other chemicals were of analytical grade or were of the highest quality commercially available.

**Cells and culture conditions.** The human bladder adenocarcinoma cell line EJ-1 and human colon adenocarcinoma cell line DLD-1 were obtained from the Health Science Research Resources Bank (Osaka, Japan). A clone of EJ-1 cells (#8) and a clone of DLD-1 cells (#1) were isolated and used in the present study. These cell lines and related cell lines were maintained at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (F12) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal calf serum (FCS; JRH

[3]To whom correspondence should be addressed.
E-mail: miyazaki@yokohama-cu.ac.jp

doi: 10.1111/j.1349-7006.2007.00502.x

Bioscience, Lenexa, KS, USA). Plastic culture dishes were purchased from Sumitomo Bakelite (Tokyo, Japan). The same medium was used throughout all of the experiments unless otherwise mentioned.

**Plasmid construction and transfection.** To overexpress IGFBP-rP1 in DLD-1 cells, total RNA was prepared from EJ-1 cells using TRIZOL (Invitrogen) according to the manufacturer's protocol. A human IGFBP-rP1 cDNA was prepared using the EJ-1-derived RNA as a template by reverse transcription–polymerase chain reaction. The IGFBP-rP1 cDNA was ligated into the *Bam*HI and *Eco*RI sites of the pSecTag2B mammalian expression vector (Invitrogen) that has a murine immunoglobulin κ-chain V-J2-C signal peptide. To suppress IGFBP-rP1 expression in EJ-1 cells using the RNA interference method, a siRNA expression vector was constructed using the piGENE tRNA Pur/*Kpn*I/*Sac*I vector (iGENE Therapeutics, Ibaraki, Japan) with the human $tRNA^{VAL}$ promoter. An IGFBP-rP1 DNA fragment encoding a target site was amplified by polymerase chain reaction and inserted downstream of the $tRNA^{VAL}$ promoter. This expression vector allows the fragment transcribed by RNA polymerase III to from a stem–loop-type (hairpin-type) siRNA after transfection into target cells. The DNA fragment (5′-GGCGAGATCCG-CGGCGCGTGTGGCTGCTGCcttcctgtcaGCAGCAGCCGCAC GCGTCGCGGGTCTCGCC-3′) contained sequences corresponding to a 30-nucleotide sequence from the human IGFBP-rP1 gene, a 10-nucleotide stem-loop linker (lowercase letters), and the antisense sequence of the same 30-nucleotide sequence.

Cells were transfected with the expression vectors using Lipofectamine reagent (Invitrogen) and OPTI-MEM medium (Invitrogen). Plasmid DNA (1 μg/mL) precomplexed with the Lipofectamine reagent (6 μL/mL) was added into cell culture in the serum-reduced OPTI-MEM medium. After a 6-h incubation, the medium was replaced with fresh DMEM/F12 + 10% FCS. To establish stable transfectants, the cells transfected with the pSecTag2B vector or with the piGENE tRNA Pur/*Kpn*I/*Sac*I vector were cultured in the growth medium supplemented with 100 ng/mL zeocin (Invitrogen) or 2.5 ng/mL puromycin (Sigma, St Louis, MO, USA), respectively.

**Preparation of conditioned medium.** Cells were grown to confluence in a 90-mm culture dish containing the serum-containing medium. The culture was rinsed three times with serum-free DMEM/F12, and further incubated in serum-free DMEM/F12. After incubation for 2 days, the resultant conditioned medium (10 mL) was collected, clarified by centrifugation, and dialyzed against distilled water at 4°C. The dialyzed sample was then lyophilized and dissolved in 100 μL $Ca^{2+}$- and $Mg^{2+}$-free phosphate-buffered saline (PBS).

**Electrophoretic analyses.** Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out on 12.5% gels or 5–20% gradient gels (Bio-Rad, Hercules, CA, USA) under reducing or non-reducing conditions. To detect IGFBP-rP1 by immunoblotting, the proteins separated on the gels were transferred to nitrocellulose membranes. The membranes were then blocked with 5% (w/v) skim milk overnight at 4°C. The blotted membranes were reacted sequentially at room temperature with the anti-IGFBP-rP1 monoclonal antibody #88 (0.2 μg/mL IgG) for 2 h, the biotinylated anti-mouse-IgG antibody (Vector Laboratories, Burlingame, CA, USA) for 1 h, and the alkaline phosphatase-conjugated avidin D (Vector Laboratories) for 1 h. After each incubation step the membranes were washed five times with 0.1% Tween-20 (Sigma) in PBS. Finally, immunoreactive signals were visualized by the color reaction with the substrate combination of 5-brom-4-chlor-3-indolyl-phosphate (Sigma) and nitro-blue-tetrazolium (Wako Pure Chemicals, Osaka, Japan). The reaction was stopped by washing with water.

**Tumor formation *in vivo*.** DLD-1 and EJ-1 transfectants were grown in the growth medium, harvested by trypsin treatment, and suspended in 1% FCS-containing medium. The cell suspension containing $1 \times 10^7$ cells was injected intraperitoneally (i.p.) or subcutaneously (s.c.) into male athymic mice (BALB/c-nu/nu; Charles River Japan, Kanagawa, Japan). Tumor sizes on the backs of mice were measured every 2 days after the tumors became visible. Tumor weights were measured after the mice were killed at the indicated time.

**Assay of cell growth under anchorage-dependent and -independent conditions.** For the cell growth assay in monolayer cultures, cells were seeded on 35-mm culture dishes at a density of $1 \times 10^4$ cells/dish in the DMEM/F12 + 10% FCS medium and incubated for the indicated periods of time. To assay cell growth under serum-free culture conditions, cells were first seeded and incubated overnight in the serum-containing medium, washed twice with PBS, and further incubated in the serum-free DMEM/F12 medium. In each experiment, the medium was changed to fresh medium every 2 days. After incubation, the cells were harvested by trypsinization, and the number of cells was determined using a hemocytometer. Anchorage-independent cell growth was assayed by analyzing the formation of colonies in soft agar culture. Single cells were suspended in the DMEM/F12 + 10% FCS medium containing 0.3% (w/v) Agar Noble (BD, Baltimore, MD, USA) and inoculated at a cell density of $5 \times 10^2$ EJ-1 cells/dish or $2.5 \times 10^4$ DLD-1 cells/dish on solidified, 0.5% agar-containing medium in 35-mm dishes. After 14 days incubation, the cell colonies were stained with 1 mg/mL *p*-indonitrotetrazolium violet for 24 h, and the stained cell colonies were photographed and counted.

**Cell adhesion assay.** A cell adhesion assay was carried out as described previously.[25] Briefly, each well of 96-well enzyme-linked immunosorbent assay plates (Costar, Cambridge, MA, USA) was coated with a substrate protein (laminin-5 or fibronectin) at 4°C overnight and blocked with 1% (w/v) bovine serum albumin (BSA). Cells were suspended in serum-free medium at a density of $2 \times 10^5$ cells/mL, and a 100-μL aliquot of the cell suspension was inoculated into each well of the plates and incubated at 37°C for 1 h. After non-adherent cells were removed, adherent cells were fixed and stained with Hoechst 33432 (Sigma). The fluorescence intensity of each well of the plates was measured using a CytoFluor 2350 fluorometer (Millipore, Billerica, MA, USA).

**Insulin receptor substrate-1 (IRS-1) phosphorylation.** EJ-1 and DLD-1 transfectants were grown to semiconfluence in 150-mm dishes and shifted to serum-free medium. At 48 h, these cells were stimulated with 10 μg/mL or 0.1 μg/mL insulin for 30 min at 37°C. The cells were washed twice with ice-cold PBS and lysed in a lysis buffer consisting of 1% Triton X-100, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM ethylenediaminetetracetic acid, 2 mM $Na_3VO_4$, and a protease inhibitor mixture (100 μM 4-[2-aminoethyl]-benzenesulfonylfluoride hydrochloride, 80 nM aprotinin, 5 μM bestatin, 1.5 μM E-64, 2 μM leupeptin and 1 μM pepstatin) (Wako Pure Chemicals, Osaka, Japan). Solubilized proteins were immunoprecipitated with anti-insulin receptor substrate-1 antibody (Upstate Biotechnology, Lake Placid, NY, USA) and subjected to immunoblotting as described above. Protein tyrosine phosphorylation was detected with an anti-phosphotyrosine monoclonal antibody (Transduction Laboratories, Lexington, KY, USA) at 1:1000 dilution. The same polyvinylidene fluoride membrane (Millipore) was reprobed with the anti-IRS-1 antibody. Immunoreactive proteins were visualized using the ECL detection system (Amersham, Buckinghamshire, UK).

**Immunofluorescence staining of IGFBP-rP1.** Cultured cells were fixed with 10% formalin at room temperature for 20 min. They were treated with 0.1% Triton X-100 at room temperature for 15 min to permeabilize membranes, then incubated at 4°C overnight in a blocking buffer (1.2% BSA in PBS). The cells were incubated in the blocking buffer supplemented with the anti-IGFBP-rP1 monoclonal antibody #88 (2 μg/mL IgG) at room


doi: 10.1111/j.1349-7006.2007.00502.x

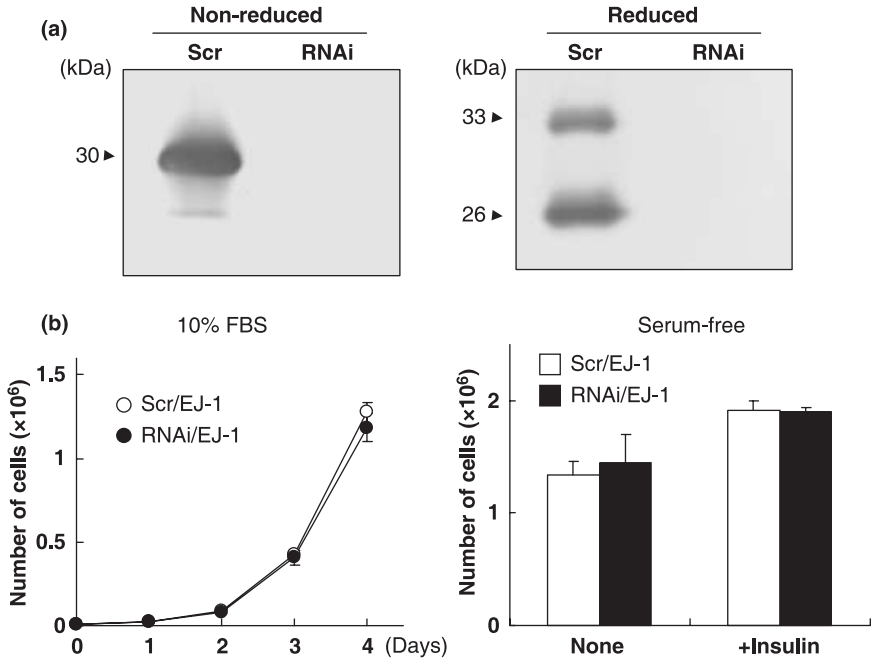


**Fig. 1.** Effects of introducing an insulin-like growth factor-binding protein-related protein 1 (IGFBP-rP1) RNA interference (RNAi) vector into EJ-1 human bladder carcinoma cells. An IGFBP-rP1 RNAi vector and a control vector were transfected into EJ-1 cells, and the cells maintaining the introduced genes, RNAi/EJ-1 and Scr/EJ-1, respectively, were selected with puromycin. (a) Analysis of IGFBP-rP1 secreted by transfected cells. Serum-free conditioned medium was collected from confluent cultures of Scr/EJ-1 (Scr) and RNAi/EJ-1 (RNAi) cells, concentrated 100-fold, and subjected to immunoblotting with the anti-IGFBP-rP1 monoclonal antibody #88 under non-reducing (left panel) and reducing (right panel) conditions. (b) Comparison of cell growth rates. Scr/EJ-1 and RNAi/EJ-1 cells were inoculated in 10% fetal calf serum-containing medium (left panel) or serum-free basal medium (right panel) without (None) or with 10 μg/mL insulin (+Insulin) at a density of $1 \times 10^4$ cells/35-mm dish, and incubated for the indicated lengths of time (11 days for the serum-free cultures). After the incubation, grown cells were counted. Each point represents the mean ± SD of the number of cells in triplicate dishes.

temperature for 1 h. After the first antibody was washed, the IGFBP-rP1-bound antibody was visualized with goat antimouse-IgG antibody conjugated with fluorescein-isothiocyanate (Vector Laboratories). Fluorescence images were obtained using a fluorescence microscope (KEYENCE, Osaka, Japan).

**Preparation of Dynabeads conjugated with anti-IGFBP-rP1 antibody or purified IGFBP-rP1 protein.** To detect IGFBP-rP1 bound to the cell surface, Dynabeads M-280 (Dynal Biotech, Oslo, Norway) were used. Dynabeads that had been conjugated with sheep anti-mouse-IgG antibody were bound to the anti-IGFBP-rP1 mouse monoclonal antibody (#88) according to the manufacturer's instructions. Briefly, the anti-IGFBP-rP1 antibody (27 μg/mL) was incubated with anti-mouse-IgG Dynabeads M-280 ($10^7$ beads) at 4°C for 18 h with slow rotation. The resultant antibody-bound beads were washed three times with PBS and used. To conjugate IGFBP-rP1 protein to Dynabeads, the anti-IGFBP-rP1 antibody-bound beads were incubated with purified IGFBP-rP1 (20 μg) at 4°C for 18 h, washed three times with PBS, and used to detect a cell surface receptor of IGFBP-rP1.

## Results

**Suppression of IGFBP-rP1 expression in EJ-1 cells.** IGFBP-rP1 is expressed in many kinds of human cancer cell lines as well as in normal fibroblast lines. The bladder carcinoma cell line EJ-1 expresses IGFBP-rP1 at a high level, but the colon carcinoma cell line DLD-1 does not express it at all.[26] To investigate the roles of IGFBP-rP1 in tumor growth, we chose two human cancer cell lines, EJ-1 and DLD-1.

To examine whether IGFBP-rP1 affects the tumor formation of EJ-1 cells, we first attempted to suppress IGFBP-rP1 synthesis in EJ-1 cells using RNA interference. Either an siRNA expression vector or a control vector expressing a scrambled RNA was introduced into EJ-1 cells, and stable transfectants, named RNAi/EJ-1 and Scr/EJ-1, respectively, were established. The production of IGFBP-rP1 by the two cell lines was analyzed by immunoblotting with anti-IGFBP-rP1 antibody (Fig. 1a). The conditioned medium of Scr/EJ-1 cells showed a strong IGFBP-rP1 band at 30-kDa under non-reducing conditions. When the same sample was analyzed under reducing conditions, the IGFBP-rP1 protein was separated into a 33-kDa native form and a 26-kDa proteolytically cleaved form.[13,26] In contrast, IGFBP-rP1 was scarcely detected in the conditioned medium of RNAi/EJ-1 cells under both reducing and non-reducing conditions, indicating that IGFBP-rP1 production was effectively suppressed by the RNA interference.

When the growth rates of RNAi/EJ-1 and Scr/EJ-1 cells were compared in monolayer cultures with 10% FCS-containing medium (Fig. 1b, left panel) or serum-free medium (Fig. 1b, right panel), there was no significant difference between their growth rates under either of the conditions. Addition of insulin into the serum-free cultures significantly increased the cell growth rates, but again there was no difference between the growth rates of RNAi/EJ-1 and Scr/EJ-1 cells (Fig. 1b, right panel).

**Effect of IGFBP-rP1 knockdown on tumor growth *in vivo*.** To investigate the effect of IGFBP-rP1 knockdown on tumor growth *in vivo*, the two EJ-1 transfectants (Scr/EJ-1 and RNAi/EJ-1) were injected i.p. into BALB/c–nu/nu mice. When the mice were killed 14 days after injection, no visible tumors were found in the mice injected with Scr/EJ-1 cells (Fig. 2a,b). In contrast to Scr/EJ-1 cells, RNAi/EJ-1 cells produced many tumor nodules in the abdominal cavity of mice, especially on the mesentery and intestinal tract.

When injected s.c. onto the back of mice, Scr/EJ-1 cells produced small tumors, whereas RNAi/EJ-1 cells produced much larger tumors. The average weight of tumors was 0.23 ± 0.11 g for the Scr/EJ-1 tumors and 1.02 ± 0.31 g for the RNAi/EJ-1 tumors ($P < 0.001$) (Fig. 2c,d). These results clearly indicate that the tumorigenicity of EJ-1 cells is markedly increased by the suppression of IGFBP-rP1 expression by siRNA.

**Effect of exogenous IGFBP-rP1 gene expression on the tumor growth of DLD-1 cells.** In contrast to EJ-1 cells, the human cancer cell line DLD-1 does not secrete endogenous IGFBP-rP1 at a detectable level.[13] To examine the effect of IGFBP-rP1 expression on tumor growth, an IGFBP-rP1 expression vector (IGFBP-rP1/pSecTag2B) or the control vector (pSecTag2B) was introduced into DLD-1 cells, and stable transfectants were selected by zeocin. From the IGFBP-rP1 transfectant, named rP1/DLD-1, three cell clones expressing IGFBP-rP1 at high

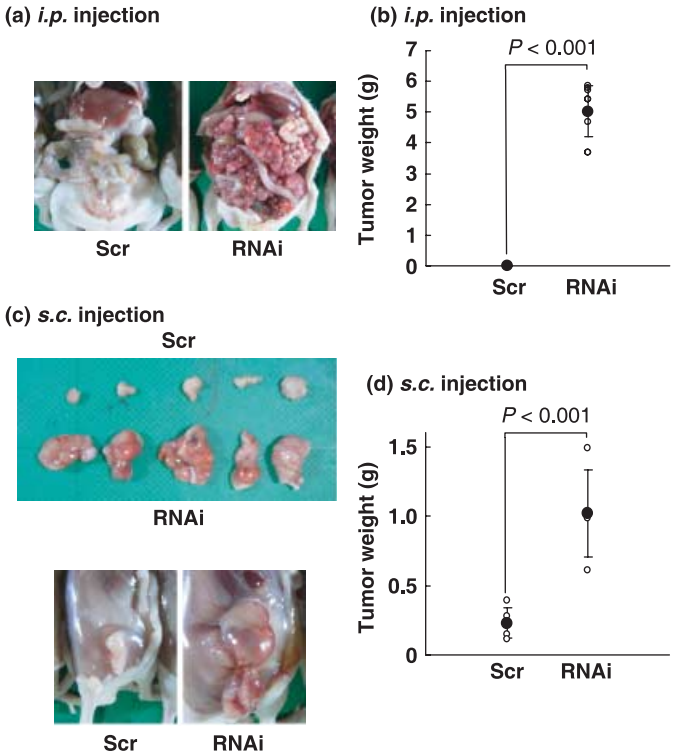


**Fig. 2.** Tumor growth of Scr/EJ-1 and RNAi/EJ-1 cells in nude mice. Scr/EJ-1 (Scr) and RNAi/EJ-1 (RNAi) cells were (a,b) intraperitoneally (i.p.) or (c,d) subcutaneously (s.c.) injected into nude mice at a dose of $1 \times 10^7$ cells/mouse (i.p., $n = 8$; s.c., $n = 5$). At 20 days after the i.p. injection or 24 days after the s.c. injection, photographs of the mice and tumors were taken (a,c), and the tumor weights were measured (b,d). Typical examples of mice are shown in (a) and the lower panel of (c). Closed circles represent the mean of total tumor weights (g) ± SD (bar) in each group: (b) 0 (Scr, $n = 8$) versus 5.02 ± 0.84 (RNAi, $n = 8$), $P < 0.001$; (d) 0.23 ± 0.11 (Scr, $n = 5$) versus 1.02 ± 0.31 (RNAi, $n = 5$), $P = 0.0016$.

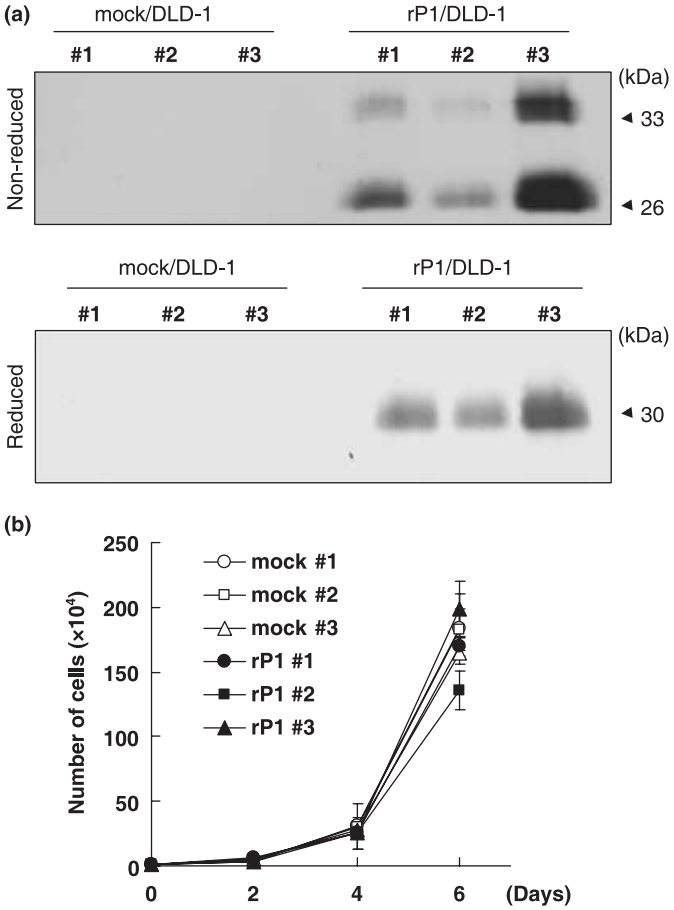


**Fig. 3.** Effects of overexpression of the exogenous insulin-like growth factor-binding protein-related protein 1 (IGFBP-rP1) gene in human colon cancer cell line DLD-1 on cell growth. An IGFBP-rP1 expression vector and a control vector were transfected into DLD-1 cells, and the cells maintaining the introduced genes, named rP1/DLD-1 and mock/DLD-1, respectively, were selected with zeocin. Three clones (#1–3) were isolated from each cell line. (a) Analysis of IGFBP-rP1 secreted by transfected cells. IGFBP-rP1 secreted into culture medium by each cell clone was analyzed by immunoblotting with the anti-IGFBP-rP1 antibody under non-reducing (upper panel) and reducing (lower panel) conditions. (b) Comparison of cell growth rates. Three cell clones from each of mock/DLD-1 and rP1/DLD-1 cell lines were inoculated at a density of $1 \times 10^4$ cells/35-mm dish in the 10% fetal calf serum-containing medium and incubated for the indicated lengths of time. After the incubation, grown cells were counted. Each point represents the mean ± SD of the numbers of cells in triplicate dishes.

levels were isolated by the limiting dilution method. Three clones were also obtained from the control DLD-1 transfectant, named mock/DLD-1, which had been transfected with the pSecTag2B control vector. The conditioned media of these clones were subjected to immunoblotting for IGFBP-rP1 detection under non-reducing and reducing conditions. As shown in Fig. 3a, none of the three mock/DLD-1 clones showed any immunoreactive band, whereas the three rP1/DLD-1 cell clones showed high levels of the 33-kDa native IGFBP-rP1 and the 26-kDa cleaved form.

When the cell growth rates of DLD-1 transfectants in monolayer culture were compared, there was no significant difference between the rP1/DLD-1 and mock/DLD-1 groups in the 10% FCS-containing growth medium (Fig. 3b). To examine the effect of IGFBP-rP1 expression on tumor growth, rP1/DLD-1 cell clones (#1–3) and mock/DLD-1 cell clones (#1–3) were injected i.p. or s.c., respectively, into nude mice, and the weights of tumors were compared between the two groups 45 days after implantation (Fig. 4). With the i.p. injection, all three of the mock/DLD-1 clones produced large tumor nodules in the abdominal cavity, whereas the mock/DLD-1 clones produced far fewer and smaller tumors: the overall average weight of the tumors was 3.30 ± 0.78 g for mock/DLD-1 and 0.60 ± 0.53 g for rP1/DLD-1 ($P < 0.001$) (Fig. 4a,b). Similar but smaller differences were obtained with the s.c. injection: the average weight of the tumors was 1.19 ± 0.57 g for the three clones of mock/DLD-1, and 0.40 ± 0.47 g for the three clones of rP1/DLD-1 ($P < 0.001$) (Fig. 4c,d). These results indicate that the expression of IGFBP-rP1 in DLD-1 cells markedly decreases the tumorigenicity.

**Anchorage-independent cell growth.** To understand the reason for the differential tumor growth rates between IGFBP-rP1-expressing and non-expressing cancer cell lines, their properties were further investigated in culture.

It has long been known that the ability of mammalian cells to proliferate independently of anchorage *in vitro* often correlates with their ability to form tumors *in vivo*. We therefore first compared the abilities of Scr/EJ-1 and RNAi/EJ-1 cells to grow and form colonies in soft agar culture. After 2 weeks in the soft agar culture, Scr/EJ-1 cells formed few colonies, whereas RNAi/EJ-1 cells formed many colonies (Fig. 5a). To test whether exogenous IGFBP-rP1 suppressed colony formation, RNAi/EJ-1 cells were incubated in the presence of purified IGFBP-rP1 in the soft agar medium. The exogenous IGFBP-rP1 weakly but significantly suppressed the colony formation ($P < 0.05$) (Fig. 5b).


doi: 10.1111/j.1349-7006.2007.00502.x

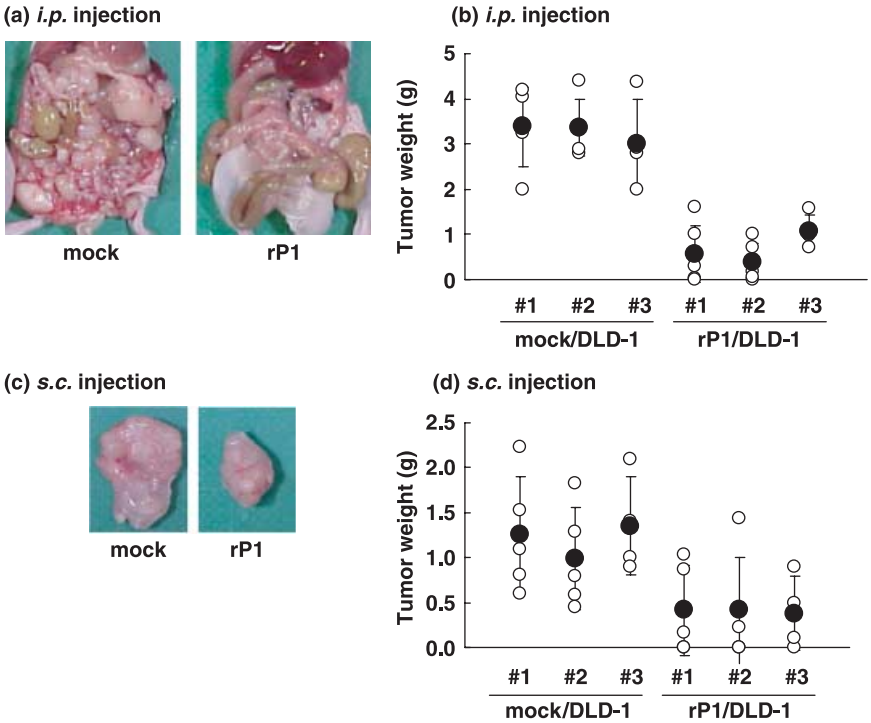


**Fig. 4.** Effect of insulin-like growth factor-binding protein-related protein 1 (IGFBP-rP1) expression in DLD-1 colon cancer cells on tumor growth in nude mice. Three clones of the IGFBP-rP1-expressing cell line rP1/DLD-1 and the non-expressing control cell line mock/DLD-1 were injected into the (a,b) intraperitoneal (i.p.) or (c,d) subcutaneous (s.c.) region of nude mice at a dose of $1 \times 10^7$ cells/mouse (i.p., $n = 5$; s.c., $n = 5$). At 50 days after the i.p. injection or 60 days after the s.c. injection, the mice were dissected, and the total tumor weight was measured for each mouse (b,d). Typical examples of the mice and tumors are shown in photographs (a) and (c). In (b) and (d), closed circles represent the mean of total tumor weights ± SD (bar) in each group. In the i.p. xenograft, the average tumor weight (g) of each clone was, in the order of #1, #2 and #3, $3.38 \pm 0.87$, $3.36 \pm 0.64$ and $3.02 \pm 0.98$ in mock/DLD-1, and $0.56 \pm 0.63$, $0.40 \pm 0.40$ and $1.06 \pm 0.37$ in rP1/DLD-1 ($P < 0.001$). In the s.c. xenograft, the average tumor weight (g) of each clone was, in the order of #1, #2 and #3, $1.25 \pm 0.65$, $0.99 \pm 0.57$ and $1.35 \pm 0.54$ in mock/DLD-1, and $0.42 \pm 0.50$, $0.42 \pm 0.59$ and $0.38 \pm 0.41$ in rP1/DLD-1 ($P < 0.001$).

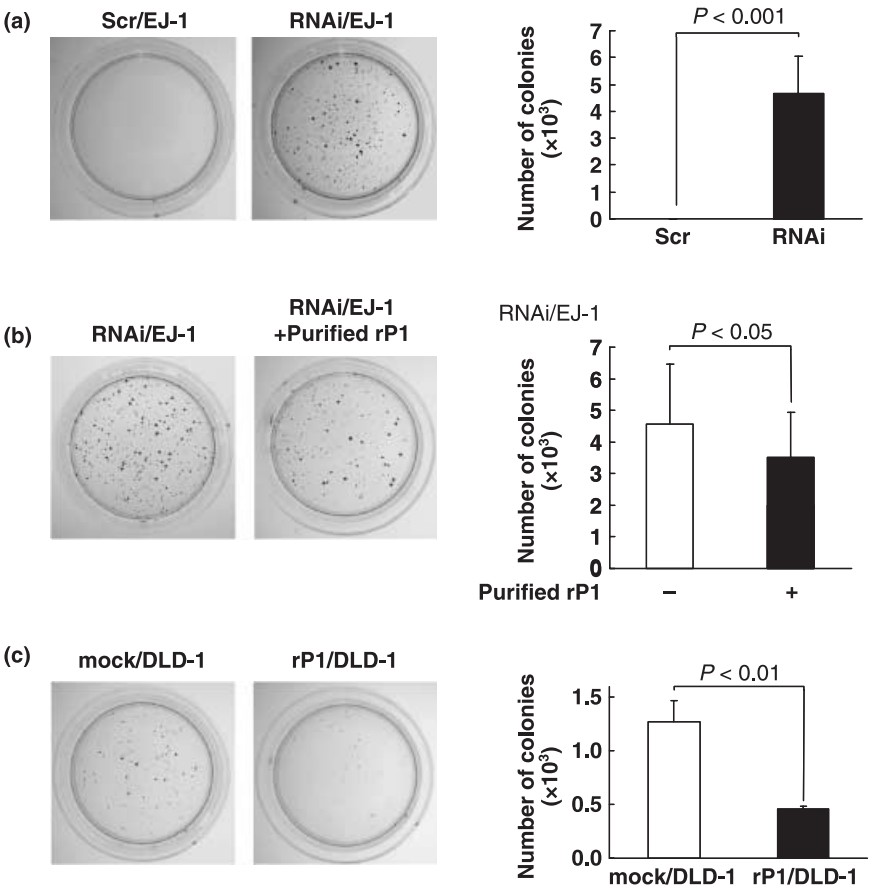


**Fig. 5.** Effects of insulin-like growth factor-binding protein-related protein 1 (IGFBP-rP1) on anchorage-independent cell growth. Anchorage-independent cell growth ability was assayed by analyzing the formation of colonies in soft agar culture. Single cells were suspended in the Dulbecco's modified Eagle's medium/Ham's F12 medium + 10% fetal calf serum medium containing 0.3% (w/v) Agar Noble, and were placed at a density of $5 \times 10^2$ EJ-1 cells/dish or $2.5 \times 10^4$ DLD-1 cells/dish on solidified 0.5% agar-containing medium in 35-mm dishes. After 14 days incubation, the cell colonies were stained with 1 mg/mL *p*-indonitrotetrazolium violet for 24 h, and cell colonies formed were photographed and counted by NIH image. Scr/EJ-1 cells (open bar) formed few colonies, whereas RNAi/EJ-1 cells (closed bar) formed many colonies (a). To test if exogenous IGFBP-rP1 suppressed colony formation, RNAi/EJ-1 cells were incubated in the presence of purified IGFBP-rP1 (20 μg/mL) in the soft agar medium. The exogenous IGFBP-rP1 weakly suppressed colony formation (b, $P = 0.02$). Mock/DLD-1 cells (#1, open bar) formed many colonies, whereas rP1/DLD-1 cells (#3, closed bar) formed far fewer colonies (c, $P = 0.01$).

The anchorage-independent growth ability was also tested with one clone each of the mock/DLD-1 and rP1/DLD-1 cells. rP1/DLD-1 cells formed far fewer colonies than mock/DLD-1 cells (Fig. 5c). These results clearly indicate that the expression of IGFBP-rP1 strongly suppresses the anchorage-independent growth ability of tumor cells.

**Effect of IGFBP-rP1 expression on cell adhesion.** It has been reported that IGFBP-rP1 weakly supports cell adhesion by

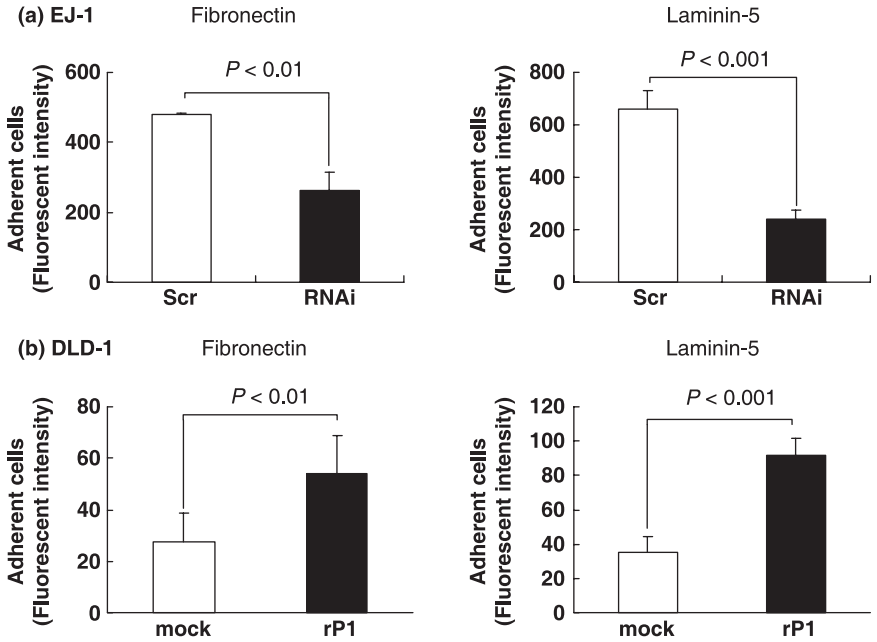


**Fig. 6.** Effects of overexpression or downregulation of insulin-like growth factor-binding protein-related protein 1 (IGFBP-rP1) on the cell adhesion activity of EJ-1 and DLD-1 cells. Cell attachment efficiency to two cell adhesion substrates (fibronectin and laminin-5) was compared between (a) Scr/EJ-1 (Scr) and RNAi/EJ-1 (RNAi) cells and between (b) mock/DLD-1#1 (mock) and rP1/DLD-1#3 (rP1) cells. Cells suspended in serum-free culture medium were seeded onto 96-well plates precoated with 2.0 μg/mL human fibronectin (left panels) or 0.5 μg/mL human laminin-5 (right panels) and incubated at 37°C for 1 h. Cells attached to the plates were stained with Hoechst 33432 and measured for absorbance at 590 nm. Each point represents the mean ± SD of the $A_{590}$ values in triplicate dishes.

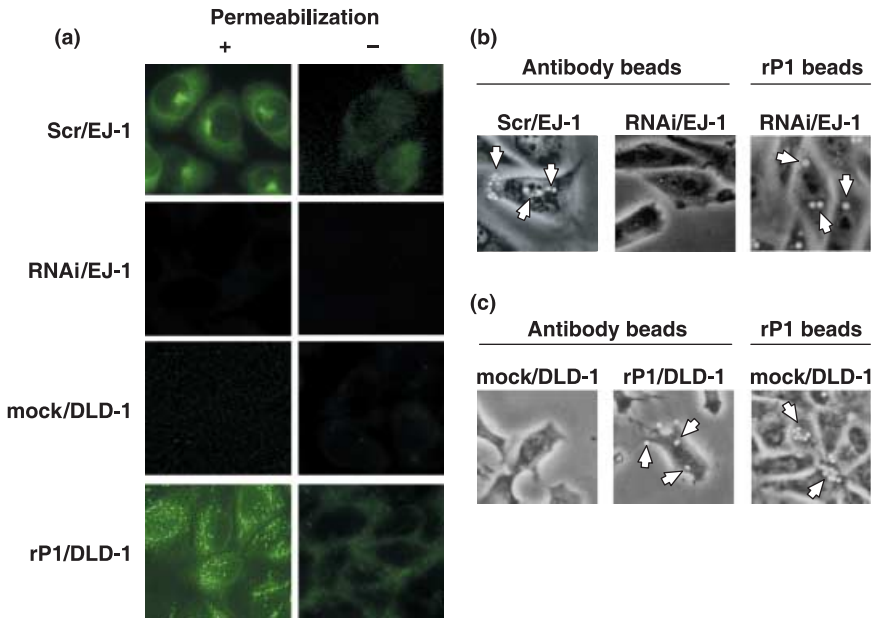


**Fig. 7.** Binding of insulin-like growth factor-binding protein-related protein 1 (IGFBP-rP1) to the cell surface. (a) Immunostaining of IGFBP-rP1 bound to IGFBP-rP1-expressing cells. The two sets of cell lines, Scr/EJ-1 (Scr) and RNAi/EJ-1 (RNAi) (upper four panels) and mock/DLD-1#1 and rP1/DLD-1#3 (lower four panels), were incubated in the fetal calf serum-containing medium, fixed with 10% formalin and treated with (left panels) or without (right panels) 0.1% Triton X-100 to permeabilize the cell membranes. The cells were then stained with the anti-IGFBP-rP1 monoclonal antibody #88. The bound primary antibody was visualized with fluorescein-isothiocyanate-conjugated secondary antibodies. (b,c) IGFBP-rP1 and its receptor on the cell surface were detected with Dynabeads linked with the IGFBP-rP1 antibody (antibody beads) or the IGFBP-rP1 protein (rP1 beads). (b) Scr/EJ-1 and RNAi/EJ-1 cells and (c) mock/DLD-1#1 and rP1/DLD-1#3 cells were allowed to spread for 24 h under standard culture conditions, and the anti-IGFBP-rP1 antibody-bound beads (left two panels) or IGFBP-rP1-bound beads (right panel) were then added to the cultures. After incubation with the beads for 24 h, photographs were taken under a phase-contrast microscope. Arrows indicate beads attached to the cell membrane.

interacting with cell-surface syndecans.[10–12] In the present study, we compared the cell adhesion efficiency of the pairs of EJ-1 and DLD-1 cell lines to fibronectin and laminin-5. These cells were suspended in serum-free medium and inoculated on plastic plates that had previously been coated with fibronectin or laminin-5. RNAi/EJ-1 cells attached poorly to both fibronectin and laminin-5 compared with the high IGFBP-rP1 producer Scr/EJ-1 (Fig. 6a). When one clone each of the mock/DLD-1 and rP1/DLD-1 cells were compared, rP1/DLD-1 cells attached to laminin-5 and fibronectin more efficiently than mock/DLD-1 cells (Fig. 6b). These results show that the IGFBP-rP1 expression increases cell adhesion activity in both EJ-1 and DLD-1 models.

The higher cell adhesion activity of the IGFBP-rP1-expressing cells compared to the non-producers suggests that endogenous IGFBP-rP1 binds to the cell surface, promoting cell adhesion. To test this possibility, IGFBP-rP1 on the cell surface was analyzed by immunofluorescence microscopy with anti-IGFBP-rP1 monoclonal antibody #88. When cells were immunostained without permeabilization, an immunofluorescent signal was evident on Scr/EJ-1 and rP1/DLD-1 cells, but little signal was detected on RNAi/EJ-1 and mock/DLD-1 cells, suggesting that IGFBP-rP1 is present on the cell surface (Fig. 7a, right panels). When cells were permeabilized, Golgi-like vesicles were stained strongly in Scr/EJ-1 and rP1/DLD-1 cells (Fig. 7a, left panels).

To further confirm the cell-surface binding of IGFBP-rP1, we used Dynabeads that had been linked with the anti-IGFBP-rP1 antibody or the purified IGFBP-rP1 protein (Fig. 7b,c). The antibody-linked beads efficiently bound to the cell surface of Scr/EJ-1 and rP1/DLD-1 cell lines, but they scarcely bound to RNAi/EJ-1 and mock/DLD-1 cells. Furthermore, the


doi: 10.1111/j.1349-7006.2007.00502.x

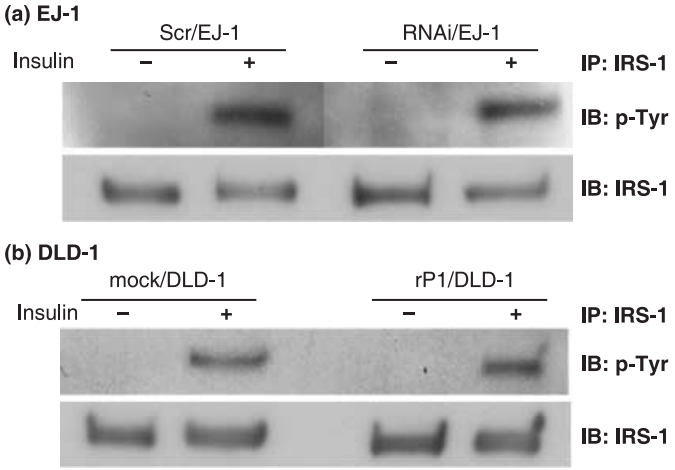


**Fig. 8.** Effect of insulin-like growth factor-binding protein-related protein 1 (IGFBP-rP1) expression on the insulin-induced phosphorylation of insulin receptor substrate-1 (IRS-1). Scr/EJ-1 and RNAi/EJ-1 cells (a) and mock/DLD-1#1 and rP1/DLD-1#3 cells (b) were incubated overnight in a serum-free basal medium, and the serum-starved cells were stimulated with 0.17 nM insulin for 30 min. From the resultant cultures, cell lysates were prepared and immunoprecipitated with an anti-IRS-1 antibody. The immunoprecipitates were immunoblotted with an antiphosphotyrosine antibody (p-Tyr) or the anti-IRS-1 antibody (IRS-1).

IGFBP-rP1-linked beads were able to bind to the surface of RNAi/EJ-1 and mock/DLD-1, which poorly or scarcely produced endogenous IGFBP-rP1 (Fig. 7b,c, right panels). These results indicate that both cell lines had a receptor for IGFBP-rP1, and the IGFBP-rP1-expressing cells had IGFBP-rP1 molecules bound to the cell surface.

**Insulin-induced signal transduction.** Past studies have shown that IGFBP-rP1 binds insulin and IGF weakly.[6,7] Therefore, it is conceivable that IGFBP-rP1 suppresses tumor growth by interfering with insulin or IGF signaling. To test this possibility, we examined the effect of IGFBP-rP1 on the phosphorylation of IRS-1, which mediates insulin receptor signaling. In the four cell lines tested, insulin increased the phosphorylation of IRS-1 (Fig. 8). However, there was no significant difference in IRS-1 phosphorylation between Scr/EJ-1 and RNAi/EJ-1 and between mock/DLD-1 and rP1/DLD-1. This suggests that IGFBP-rP1 expression does not affect insulin receptor signaling.

## Discussion

Previous studies have shown that expression of IGFBP-rP1 suppresses proliferation of breast and prostate cancer cells, and induces their cellular senescence or apoptosis *in vitro*.[18,21,27] In addition, it has been reported that IGFBP-rP1 expression is inversely associated with tumor progression in human cancers.[19] However, there was little evidence that IGFBP-rP1 suppresses tumor growth *in vivo*. Very recently, Chen *et al*. showed that the introduction of an exogenous IGFBP-rP1 gene into a human lung cancer cell line suppressed colony-forming ability and tumor growth rate.[28] In the present study with two models of human cancer cell lines, we demonstrated that IGFBP-rP1 expression markedly suppresses tumor growth *in vivo*. The IGFBP-rP1-expressing bladder carcinoma cell line Scr/EJ-1 grew poorly in nude mice after both i.p. and s.c. injection, whereas the IGFBP-rP1 knockdown cell line RNAi/EJ-1 grew rapidly and produced large tumors. Similarly, the control colon cancer cell line mock/DLD-1, which did not express endogenous IGFBP-rP1, grew rapidly *in vivo*, whereas its IGFBP-rP1-expressing counterpart rP1/DLD-1 grew very slowly.

The present findings strongly support the hypothesis that IGFBP-rP1 is a tumor-suppressive protein. However, there are some inconsistencies between present and past studies. Although past studies have suggested that IGFBP-rP1 has growth-inhibitory effects on mammary and prostate carcinoma cells in standard monolayer cultures, we could see no significant difference in growth and apoptosis between IGFBP-rP1-expressing and non-expressing cell lines of EJ-1 and DLD-1 cells. Therefore, the suppressed tumorigenicity of IGFBP-rP1-expressing cancer cells did not result from their suppressed growth ability, at least for these cell lines. However, IGFBP-rP1-expressing EJ-1 and DLD-1 cells showed a very low level or lack of anchorage-independent growth ability compared with their respective IGFBP-rP1-non-expressing counterparts. Thus, the anchorage-independent growth ability of EJ-1 and DLD-1 cells was associated with growth ability *in vivo*. It is generally accepted that binding of integrins to extracellular matrix proteins induces intracellular signaling that prevents apoptosis and supports cell survival.[29] Tumor cells are often resistant to cell apoptosis under anchorage-independent conditions, namely anoikis. The anchorage-independent growth ability is one of the most representative phenotypes of malignantly transformed cells and is well correlated with tumorigenicity. However, the mechanism of anchorage-independent cell growth is poorly understood. It is supposed that elevated growth factor signaling reduces the necessity of integrin-mediated signal transduction from cell adhesion substrates.[30] This may be true in the case of cell lines whose growth is suppressed by IGFBP-rP1 expression under anchorage-dependent growth conditions.[18,27] However, this is unlikely for EJ-1 and DLD-1 cells because their growth was not affected by IGFBP-rP1 expression under anchorage-dependent culture conditions.

IGFBP-rP1 was originally discovered as a cell adhesion factor (TAF) secreted by EJ-1 cells.[9] It promotes weak cell adhesion, probably by binding cell surface heparan sulfate proteoglycans.[11] In accordance with the cell adhesion activity of IGFBP-rP1, IGFBP-rP1-expressing EJ-1 and DLD-1 cells more efficiently adhered to fibronectin and laminin-5 than their IGFBP-rP1-non-expressing counterparts. The cell adhesion activity of cancer cells was inversely correlated with their anchorage-independent growth ability. Treatment of the IGFBP-rP1-non-expressing EJ-1 cells with purified IGFBP-rP1 weakly inhibited their anchorage-independent growth. Furthermore, we found that IGFBP-rP1 was present on the cell surface. These results suggest the hypothesis that IGFBP-rP1 on the cell surface favors cell adhesion but inhibits the anchorage-independent growth of tumor cells, thereby suppressing tumorigenicity *in vivo*. The difference in tumor growth between the IGFBP-rP1-expressing cells and the IGFBP-rP1-non-expressing counterparts was more prominent with the i.p. injection than with the s.c. injection. This may also support the above-mentioned hypothesis. However, this hypothesis and its molecular mechanism remain to be investigated in more detail.

Some previous studies have shown decreased expression of IGFBP-rP1 in breast cancers[19] and prostate cancers[20] compared with normal corresponding tissues, whereas other studies have shown increased expression of IGFBP-rP1 in colon cancer tissues,[31] especially at the invasion front.[14] At present this discrepancy can not be explained. It is also unknown whether the IGFBP-rP1 expressed by cancer cells acts as a tumor-suppressive factor. However, it seems possible that the tumor-suppressive action of IGFBP-rP1 is blocked or modulated by other factors. We have reported that IGFBP-rP1 is cleaved by matriptase, changing its biological activities.[13,26] In addition, we recently found that IGFBP-rP1 is heterogeneous with respect to glycosylation and heparin-binding activity (Y. Sato, Z. Chen and K. Miyazaki, unpublished data). These modifications of IGFBP-rP1 are likely to regulate the tumor-suppressive activity of

IGFBP-rP1. It should also be noted that IGFBP-rP1 is highly expressed in blood vessels in tumor tissues[6] and high endothelial venule cells in lymphoid tissues.[32] IGFBP-rP1 is known to interact with many factors besides insulin and IGFs, such as type IV collagen,[6] syndecans or heparansulfates,[11–13] chemokines and interferon-γ-inducible protein 10.[33] Moreover, IGFBP-rP1 stimulates prostacyclin production.[7] It is conceivable that such specific localization and biological activities of IGFBP-rP1 also modulate the tumor-suppressive activity of IGFBP-rP1.

IGFBPs bind IGFs with high affinity and thereby regulate the amount of IGFs available for binding its receptors. Interestingly, IGFBPs are known to modulate cellular proliferation or apoptosis not only by the IGF-dependent mechanism but also by an IGF-independent mechanism.[1] For example, IGFBP-3 acts as a competitive inhibitor of IGF action and inhibits the proliferation of IGF-sensitive cells.[34] IGFBP-3 also inhibits the proliferation of IGF-insensitive cells, probably by interacting with an unknown cell surface receptor or by acting as a nuclear transcription factor.[35,36] IGFBP-4 inhibits the proliferation of IGF-sensitive colon cancer cells under both anchorage-dependent and -independent conditions, whereas it inhibits the proliferation of IGF-insensitive colon cancer cells only under anchorage-independent conditions.[37] These findings suggest that IGFBP regulate cellular proliferation by multiple mechanisms, depending on cell types and experimental conditions.[1] Compared with IGFBPs, IGFBP-rP1 has a very low binding affinity for IGFs, though its affinity for insulin is higher than that of IGFBPs.[38] Therefore, it is not clear whether IGFBP-rP1 modulates insulin or IGF actions. In the present study, insulin activated an insulin or IGF receptor in both EJ-1 bladder cancer cells and DLD-1 colon cancer cells, as analyzed by tyrosine phosphorylation of IRS-1. However, neither suppression nor overexpression of IGFBP-rP1 affected either insulin signaling or cellular growth in these cell lines. This indicates that IGFBP-rP1 does not affect insulin signaling in these cell lines. These results imply that IGFBP-rP1 inhibits the growth of these insulin-sensitive cancer cell lines *in vivo* by an insulin- or IGF-independent mechanism.

In conclusion, the present study demonstrates that IGFBP-rP1 strongly inhibits tumor growth by an insulin- or IGF-independent mechanism. It is hypothesized that cell surface binding of IGFBP-rP1 and the resultant loss of anchorage-independent growth ability may lead to the marked loss of tumorigenicity of these human cancer cell lines. It remains to be elucidated how IGFBP-rP1 suppresses anchorage-independent growth and tumor growth.

## Acknowledgments

We thank Dr Y. Nagashima (Department of Molecular Pathology, Yokohama City University Graduate School of Medicine, Yokohama, Japan) for support with the histological analysis. We also thank T. Takechi for technical assistance, and Drs H. Yasumitsu, S. Higashi, T. Hirosaki and K. Yamamoto for helpful discussion. This work is supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

## References

1 Hwa V, Oh Y, Rosenfeld RG. The insulin-like growth factor-binding protein (IGFBP) superfamily. *Endocr Rev* 1999; **20**: 761–87.
2 Burger AM, Leyland-Jones B, Banerjee K, Spyropoulos DD, Seth AK. Essential roles of IGFBP-3 and IGFBP-rP1 in breast cancer. *Eur J Cancer* 2005; **41**: 1515–27.
3 Rechler MM. Insulin-like growth factor binding proteins. *Vitam Horm Rev* 1993; **47**: 1–114.
4 Jones JI, Clemmons DR. Insulin-like growth factors and their binding proteins: biological actions. *Endocrinol Rev* 1995; **16**: 3–34.
5 Oh Y, Nagalla SR, Yamanaka Y, Kim HS, Wilson E, Rosenfeld RG. Synthesis and characterization of insulin-like growth factor-binding protein (IGFBP)-7: Recombinant human mac25 protein specifically binds IGF-I and -II. *J Biol Chem* 1996; **271**: 30 322–5.
6 Akaogi K, Sato J, Okabe Y, Sakamoto Y, Yasumitsu H, Miyazaki K. Synergistic growth stimulation of mouse fibroblasts by tumor-derived adhesion factor with insulin-like growth factors and insulin. *Cell Growth Differ* 1996; **7**: 1671–7.
7 Yamauchi T, Umeda F, Masakado M, Isaji M, Mizushima S, Nawata H. Purification and molecular cloning of prostacyclin-stimulating factor from serum-free conditioned medium of human diploid fibroblast cells. *Biochem J* 1994; **303**: 591–8.
8 Murphy M, Pykett MJ, Harnish P, Zang KD, George DL. Identification and characterization of genes differently expressed in meningiomas. *Cell Growth Differ* 1993; **4**: 715–22.
9 Akaogi K, Okabe Y, Funahashi K *et al*. Cell adhesion activity of a 30-kDa major secreted protein from human bladder carcinoma cells. *Biochem Biophys Res Commun* 1994; **198**: 1046–53.
10 Akaogi K, Okabe Y, Sato J *et al*. Specific accumulation of tumor-derived adhesion factor in tumor blood vessels and in capillary tube-like structures of cultured vascular endothelial cells. *Proc Natl Acad Sci USA* 1996; **93**: 8384–9.
11 Sato J, Hasegawa S, Akaogi K *et al*. Identification of cell-binding site of angiomodulin (AGM/TAF/Mac25) that interacts with heparan sulfates on cell surface. *J Cell Biochem* 1999; **75**: 187–95.
12 Kishibe J, Yamada S, Okada Y *et al*. Structural requirements of heparan sulfate for the binding to the tumor-derived adhesion factor/angiomodulin that induces cord-like structures to ECV-304 human carcinoma cells. *J Biol Chem* 2000; **275**: 15 321–9.
13 Ahmed S, Yamamoto K, Sato Y *et al*. Proteolytic processing of IGFBP-related protein-1 (TAF/angiomodulin/mac25) modulates its biological activity. *Biochem Biophys Res Commun* 2003; **310**: 612–18.
14 Adachi Y, Itoh F, Yamamoto H *et al*. Expression of angiomodulin (tumor-derived adhesion factor/mac25) in invading tumor cells correlates with poor prognosis in human colorectal cancer. *Int J Cancer* 2001; **95**: 216–22.
15 Degeorges A, Wang F, Frierson HF Jr, Seth A, Sikes RA. Distribution of IGFBP-rP1 in normal human tissues. *J Histochem Cytochem* 2000; **48**: 747–54.
16 Swisshelm K, Ryan K, Tsuchiya K, Sager R. Enhanced expression of an insulin growth factor-like binding protein (mac25) in senescent human mammary epithelial cells and induced expression with retinoic acid. *Proc Natl Acad Sci USA* 1995; **92**: 4472–6.
17 Komatsu S, Okazaki Y, Tateno M *et al*. Methylation and downregulated expression of mac25/insulin-like growth factor binding protein-7 is associated with liver tumorigenesis in SV40T/t antigen transgenic mice, screened by restriction landmark genomic scanning for methylation (RLGS-M). *Biochem Biophys Res Commun* 2000; **267**: 109–17.
18 Wilson HM, Birnbaum RS, Poot M, Quinn LS, Swisshelm K. Insulin-like growth factor binding protein-related protein 1 inhibits proliferation of MCF-7 breast cancer cells via a senescence-like mechanism. *Cell Growth Differ* 2002; **13**: 205–13.
19 Burger AM, Zhang X, Li H *et al*. Down-regulation of T1A12/mac25, a novel insulin-like growth factor binding protein related gene, is associated with disease progression in breast carcinomas. *Oncogene* 1998; **16**: 2459–67.
20 Hwa V, Tomasini-Sprenger C, Bermejo AL, Rosenfeld RG, Plymate SR. Characterization of insulin-like growth factor-binding protein-related protein-1 in prostate cells. *J Clin Endocrinol Metab* 1998; **83**: 4355–62.
21 Sprenger CC, Damon SE, Hwa V, Rosenfeld RG, Plymate SR. Insulin-like growth factor binding protein-related protein 1 (IGFBP-rP1) is a potential tumor suppressor protein for prostate cancer. *Cancer Res* 1999; **59**: 2370–5.
22 Sprenger CC, Vail ME, Evans K, Simurdak J, Plymate SR. Over-expression of insulin-like growth factor binding protein-related protein-1 (IGFBP-rP1/mac25) in the M12 prostate cancer cell line alters tumor growth by a delay in G1 and cyclin A associated apoptosis. *Oncogene* 2002; **21**: 140–7.
23 Kato MV. A secreted tumour-suppressor, mac25, with activin-binding activity. *Mol Med* 2000; **6**: 126–35.
24 Kariya Y, Ishida K, Tsubota Y *et al*. Efficient expression system of human recombinant laminin-5. *J Biochem* 2004; **132**: 607–12.
25 Hirosaki T, Mizushima H, Tsubota Y, Moriyama K, Miyazaki K. Structural requirement of carboxyl-terminal globular domains of laminin alpha 3 chain for promotion of rapid cell adhesion and migration by laminin-5. *J Biol Chem* 2000; **275**: 22 495–502.
26 Ahmed S, Jin X, Yagi M *et al*. Identification of membrane-bound serine proteinase matriptase as a processing enzyme of insulin-like growth factor binding protein-related protein-1 (IGFBP-rP1/angiomodulin/mac25). *FEBS J* 2006; **273**: 615–27.


doi: 10.1111/j.1349-7006.2007.00502.x

27 Mutaguchi K, Yasumoto H, Mita K *et al*. Restoration of insulin-like growth factor binding protein-related protein 1 has a tumor-suppressive activity through induction of apoptosis in human prostate cancer. *Cancer Res* 2003; **63**: 7717–23.
28 Chen Y, Pacyna-Genqelbach M, Ye F *et al*. Insulin-like growth factor binding protein-related protein 1 (IGFBP-rP1) has potential tumour-suppressive activity in human lung cancer. *J Pathol* 2007; **211**: 431–8.
29 Guo W, Giancotti FG. Integrin signaling during tumor progression. *Nat Rev Mol Cell Biol* 2004; **5**: 816–26.
30 Yamada KM, Even-Ram S. Integrin regulation of growth factor receptors. *Nat Cell Biol* 2002; **4**: E75–6.
31 Umeda F, Ono Y, Sekiguchi N *et al*. Increased mRNA expression of a novel prostacyclin-stimulating factor in human colon cancer. *J Gastroenterol* 1998; **33**: 213–17.
32 Usui T, Murai T, Tanaka T *et al*. Characterization of mac25/angiomodulin expression by high endothelial venule cells in lymphoid tissues and its identification as an inducible marker for activated endothelial cells. *Int Immunol* 2002; **14**: 1273–82.
33 Nagakubo D, Murai T, Tanaka T *et al*. A high endothelial venule secretory protein, mac25/angiomodulin, interacts with multiple high endothelial venule-associated molecules including chemokines. *J Immunol* 2003; **171**: 553–61.
34 Salahifar H, Firth SM, Baxter RC, Martin JL. Characterization of an amino-terminal fragment of insulin-like growth factor binding protein-3 and its effects in MCF-7 breast cancer cells. *Growth Horm IGF Res* 2000; **10**: 367–77.
35 Leal SM, Huang SS, Huang JS. Interactions of high affinity insulin-like growth factor-binding proteins with the type V transforming growth factor-β receptor in mink lung epithelial cells. *J Biol Chem* 1999; **274**: 6711–17.
36 Schedlich LJ, Young TF, Firth SM, Baxter RC. Insulin-like growth factor-binding protein (IGFBP)-3 and IGFBP-5 share a common nuclear transport pathway in T47D human breast carcinoma cells. *J Biol Chem* 1998; **273**: 18 347–52.
37 Diehl D, Hoeflich A, Wolf E, Lahm H. Insulin-like growth factor (IGF)-binding protein-4 inhibits colony formation of colorectal cancer cells by IGF-independent mechanisms. *Cancer Res* 2004; **64**: 1600–3.
38 Yamanaka Y, Wilson EM, Rosenfeld RG, Oh Y. Inhibition of insulin receptor activation by insulin-like growth factor binding proteins. *J Biol Chem* 1997; **272**: 30 729–34.