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ORIGINAL ARTICLE

# Activation of overexpressed glucagon-like peptide-1 receptor attenuates prostate cancer growth by inhibiting cell cycle progression

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**Keywords**
Cell cycle, Glucagon-like peptide-1 receptor, Prostate cancer

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## ABSTRACT

**Aims/Introduction:** Incretin therapy is a common treatment for type 2 diabetes mellitus. We have previously reported an anti-prostate cancer effect of glucagon-like peptide-1 receptor (GLP-1R) agonist exendin-4. The attenuation of cell proliferation in the prostate cancer cell line was dependent on GLP-1R expression. Here, we examined the relationship between human prostate cancer severity and GLP-1R expression, as well as the effect of forced expression of GLP-1R using a lentiviral vector.

**Materials and Methods:** Prostate cancer tissues were extracted by prostatectomy and biopsy. GLP-1R was overexpressed in ALVA-41 cells using a lentiviral vector (ALVA-41-GLP-1R cells). GLP-1R expression was detected by immunohistochemistry and quantitative polymerase chain reaction. Cell proliferation was examined by growth curves and bromodeoxyuridine incorporation assays. Cell cycle distribution and regulators were examined by flow cytometry and western blotting. *In vivo* experiments were carried out using a xenografted model.

**Results:** GLP-1R expression levels were significantly inversely associated with the Gleason score of human prostate cancer tissues. Abundant GLP-1R expression and functions were confirmed in ALVA-41-GLP-1R cells. Exendin-4 significantly decreased ALVA-41-GLP-1R cell proliferation in a dose-dependent manner. DNA synthesis and G1-to-S phase transition were inhibited in ALVA-41-GLP-1R cells. SKP2 expression was decreased and p27Kip1 protein was subsequently increased in ALVA-41-GLP-1R cells treated with exendin-4. *In vivo* experiments carried out by implanting ALVA-41-GLP-1R cells showed that exendin-4 decreased prostate cancer growth by activation of GLP-1R overexpressed in ALVA41-GLP-1R cells.

**Conclusions:** Forced expression of GLP-1R attenuates prostate cancer cell proliferation by inhibiting cell cycle progression *in vitro* and *in vivo*. Therefore, GLP-1R activation might be a potential therapy for prostate cancer.

## INTRODUCTION

Anti-diabetic agents mimicking incretin action, such as dipeptidyl peptidase-4 inhibitors and glucagon-like peptide-1 receptor (GLP-1R) agonists, have emerged as one of the pivotal treatments for patients with type 2 diabetes mellitus. Incretin action is recently a focus of attention because of their direct organ-protective effects that are independent of the beneficial effects associated with their glucose-lowering effects[1]. Patients with type 2 diabetes mellitus have a higher risk of cardiovascular diseases[2] and high potential for restenosis after coronary angioplasty[3] compared with individuals without type 2 diabetes mellitus. Consequently, the potential of anti-diabetic treatments

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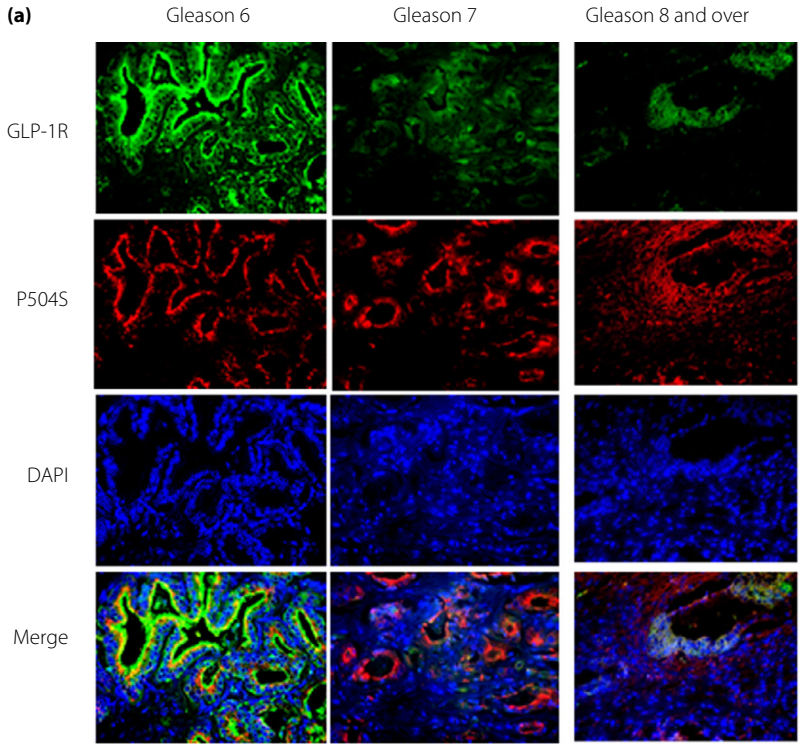
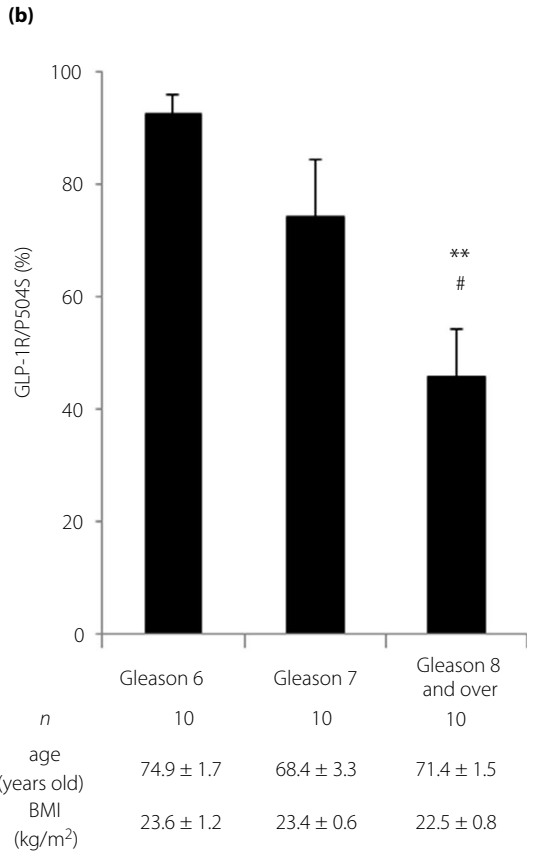
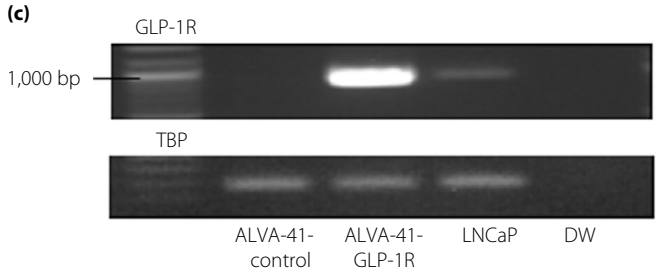
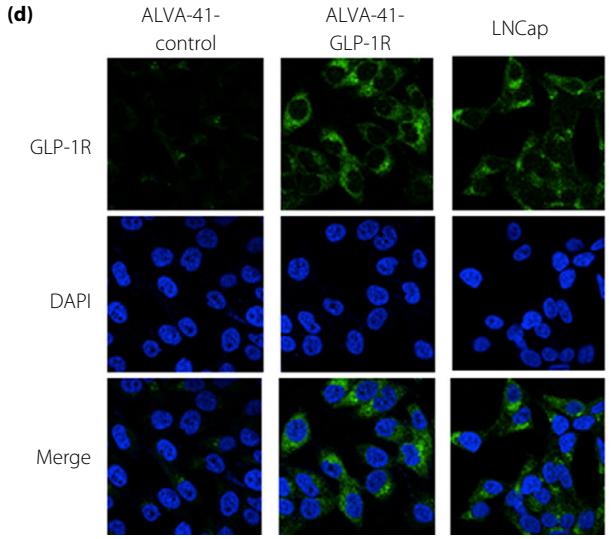



| | Gleason 6 | Gleason 7 | Gleason 8 and over |
|---|---|---|---|
| *n* | 10 | 10 | 10 |
| age (years old) | 74.9 ± 1.7 | 68.4 ± 3.3 | 71.4 ± 1.5 |
| BMI (kg/m$^2$) | 23.6 ± 1.2 | 23.4 ± 0.6 | 22.5 ± 0.8 |



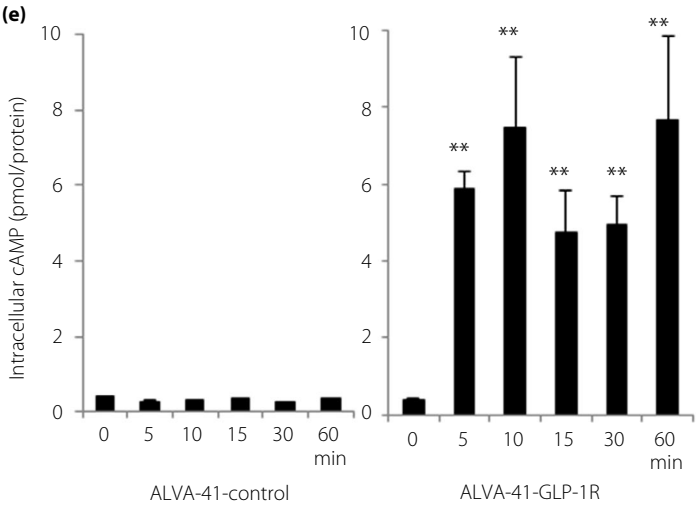



**Figure 1** | Glucagon-like peptide-1 receptor (GLP-1R) expression in human prostate cancer and overexpression of human GLP-1R in ALVA-41 cells. (a) Immunohistochemistry of GLP-1R and P504S was carried out to examine GLP-1R expression in human prostate cancer tissues obtained by prostate gland resection or biopsy. Staining is representative of prostate cancer tissues from 10 independent non-diabetic patients. Sections were stained with anti-GLP-1R or P504S antibodies and counterstained with 4′,6-diamidino-2-phenylindole (DAPI; magnification: ×400). (b) GLP-1R-positive cells and cancerous P504S-positive cells were counted in four individual fields of view. One-way ANOVA was carried out to calculate statistical significance (**$P < 0.01$ vs Gleason 6, $^{\#}P < 0.05$ vs Gleason 7). (c) Reverse transcription polymerase chain reaction of the coding sequence of human *GLP1R* was carried out to detect *GLP1R* expression. *TBP* was used as the internal control. (d) Immunohistochemistry was carried out to detect expression of human GLP-1R in ALVA-41 and LNCaP cells. (e) The intracellular cyclic adenosine monophosphate (cAMP) concentration was measured in ALVA-41-control and ALVA-41-GLP-1R cells with or without Ex-4 stimulation. The unpaired *t*-test was carried out to calculate statistical significance (**$P < 0.01$ vs 0 min; $n = 3$).

using incretin action to enable not only glycemic control, but also protection of the cardiovascular system, has been elucidated. Previously, we investigated such protective effects on vascular systems, including attenuation of atheroma formation in atherogenic mice[4] and the reduction of vascular constriction after injury[5,6], induced by a GLP-1R agonist exendin-4 (Ex-4). Thus, incretin therapy might be able to ameliorate quality of life and reduce mortality rates among patients with type 2 diabetes mellitus due to its vascular protection.

Cancer is currently a central cause of death in patients with type 2 diabetes mellitus[7]. In particular, cancer has become the leading causal disease of death in Japanese patients with type 2 diabetes mellitus. The Japan Diabetes Society and Japan Cancer Association have therefore issued a warning about the increasing cancer risk in patients with diabetes[8]. The current Japan Diabetes Optimal Integrated Treatment study for three major risk factors of cardiovascular diseases (J-DOIT3) showed that multifactorial intensive intervention reduces cardiovascular events in Japanese patients with type 2 diabetes mellitus[9]. However, J-DOIT3 did not reduce the risk of all mortalities and cancer death by multifactorial intervention[9]. This result suggests that establishing a new treatment strategy to reduce cancer and associated deaths for patients with type 2 diabetes mellitus is required.

We previously observed GLP-1R expression in postoperative prostate cancer tissue in non-diabetic individuals and showed the attenuation of prostate cancer growth by Ex-4 through inhibiting extracellular signal-related kinase (ERK) activation both *in vitro* and *in vivo*[10]. Further reductions in tumor growth and prostate cancer cell proliferation were observed by combination treatment with Ex-4 and metformin[11], without a relationship to glucose reduction. Following our experimental demonstrations, the Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results trial (LEADER), showed that the GLP-1R agonist significantly decreased the prevalence of prostate cancer in patients with type 2 diabetes mellitus, suggesting that GLP-1R agonists attenuate prostate cancer growth in not only experimental animal models, but also patients with type 2 diabetes mellitus[12]. In our previous report, the anti-prostate cancer effect induced by Ex-4 was dependent on GLP-1R expression in cancer cells, and Ex-4 did not attenuate the proliferation of the human prostate cancer cell line ALVA-41 that does not express endogenous GLP-1R[10]. To elucidate the precise anti-prostate cancer effect of GLP-1R activation, here we examined the relationship between human prostate cancer severity and GLP-1R expression, and the effect of forced expression of GLP-1R using a lentiviral vector in ALVA-41 cells *in vitro* and *in vivo*.

## METHODS

### Human tissues

Human prostate cancer tissues were obtained from 30 non-diabetic individuals with prostate cancer aged from 20 to 85 years after radical prostatectomy or transrectal biopsy in Fukuoka University Hospital, Fukuoka, Japan. The samples were embedded in paraffin, fixed in formalin and cut into 3-μm thick sections for immunohistochemical staining. Sections were prepared from 30 independent prostate cancers of 30 independent patients. The tissue samples were categorized into three malignancy grades by the Gleason grading system[13]. The Ethical Committee of Fukuoka University Hospital approved the protocol of this study (15-2-03) with opt-out consent provided by the hospital website (http://www.hop.fukuoka-u.ac.jp/rinshou/download/PDF(15-2-03).pdf). The present study was carried out in accordance with the principles of the Declaration of Helsinki.

### Immunohistochemistry

Paraffin-embedded tissue sections were stained with an anti-GLP-1R monoclonal antibody (Mab 3F52)[14] obtained from Novo Nordisk and an anti-P504S antibody (AN449-5ME; Biogenex, Fremont, CA, USA). Sections stained for GLP-1R were subsequently incubated with Alexa Fluor 488 goat anti-mouse immunoglobulin G (A-11017; Life Technologies, CA, USA), and sections stained for P504S were incubated with Alexa Fluor 594 goat anti-rabbit immunoglobulin G (A-11012; Life Technologies). The sections were counterstained with 4′,6-diamidino-2-phenylindole and then observed by microscopy.

### Construction of the lentiviral GLP-1R-expressing vector and transduction of cells

We constructed a lentiviral vector including a FLAG epitope tag as described previously[15], using the pFLAG CMV-2 expression vector (Cat. # E7033; Sigma-Aldrich, St. Louis, MO, USA),

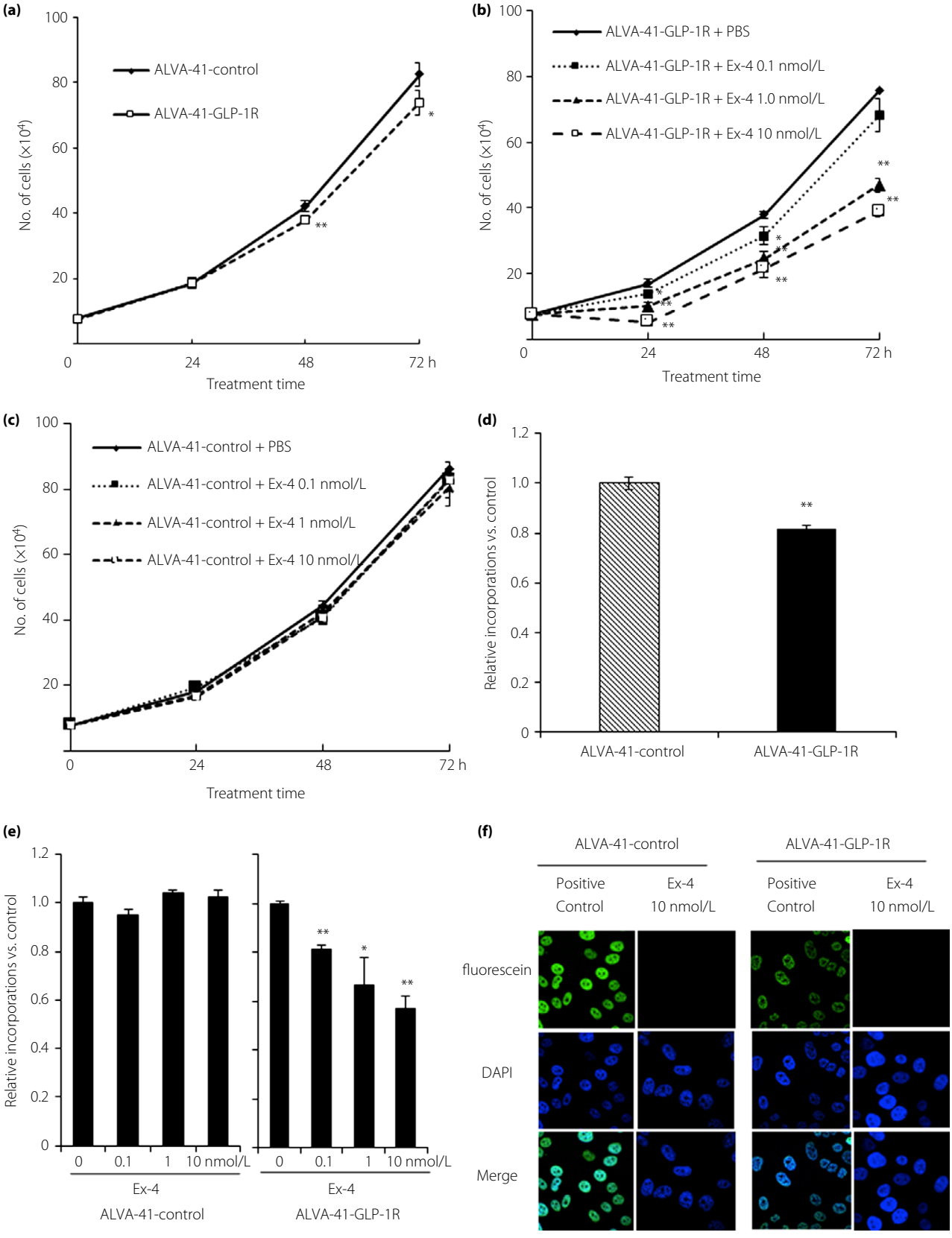


**Figure 2** | Attenuation of prostate cancer cell proliferation by overexpression of glucagon-like peptide-1 receptor (GLP-1R) and Ex-4 stimulation. Growth curves of ALVA-41-control and ALVA41-GLP-1R cells without Ex-4 A, ALVA-41-GLP-1R cells with or without Ex-4 B, and ALVA-41-control cells with or without Ex-4 C. (a) The unpaired *t*-test was carried out to calculate statistical significance (*$P < 0.05$, **$P < 0.01$ vs ALVA-control; $n = 3$). (b, c) One-way ANOVA was carried out to calculate statistical significance (*$P < 0.05$, **$P < 0.01$ vs phosphate-buffered saline; $n = 3$). (d,e) Bromodeoxyuridine assays were carried out to measure deoxyribonucleic acid synthesis in ALVA-41-control and ALVA-41-GLP-1R cells with or without Ex-4 for 24 h. Data are expressed as relative absorbance to ALVA-41-control D and 0 nmol/Lnmol/L Ex-4 in ALVA41-control or ALVA-41-GLP-1R cells. The unpaired *t*-test was carried out to calculate statistical significance. (d) *$P < 0.05$ versus ALVA-41-control ($n = 3$). (e) *$P < 0.05$, **$P < 0.01$ versus 0 nmol/L Ex-4 ($n = 3$). DAPI, 4′,6-diamidino-2-phenylindole.

pLVSIN-EF1α (Cat. #6186; Clontech, Mountain View, CA, USA) and 293T cells (#CRL-3216; ATCC, Manassas, VA, USA).

### Cell culture and proliferation assay

Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cell proliferation assay was carried out as described previously[10] with minor modifications. Briefly, cells were cultured in 12-well culture plates and maintained in medium with or without 0.1–10 nmol/L Ex-4 or 100 nmol/L exendin (9–39; E7269; Sigma-Aldrich), and with or without $PKI_{14-22}$ (P9115; Sigma-Aldrich). The cell proliferation ratio was determined after 0–3 days or 48 h using a hemocytometer.

### Animals

Male athymic CAnN.Cg-*Foxn1nu*/CrlCrlj mice, 5-weeks-old, were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) and housed in a specific-pathogen-free barrier facility at Fukuoka University. At 6 weeks-of-age, the mice were subcutaneously injected with 5,000 ALVA-41-GLP-1R or ALVA-41-control cells stably expressing the luciferase gene controlled by the CAG promoter (catalog no. LVP567; GenTarget Inc., San Diego, CA, USA), which were mixed with 100 μL Matrigel (Becton Dickinson, Bedford, MA, USA)[15]. Mice were treated with either saline or Ex-4 (Sigma-Aldrich, Tokyo, Japan), as described previously[10]. At 4 weeks after implantation, tumor growth was evaluated using an IVIS Lumina *In Vivo* Imaging System[16]. After imaging, mice were euthanized and their tumors were resected. The tumor volume was calculated as previously described[10]. The plasma glucose concentration was measured by Glutest Neo Super (Sanwa Chemical Co., Kanagawa, Japan). All protocols involving animals were reviewed and approved by the Animal Care Subcommittee at Fukuoka University. All methods involving animals were carried out in accordance with the relevant guidelines and regulations.

### Reverse transcription and quantitative real-time reverse transcription polymerase chain reaction

To analyze gene expression, reverse transcription (RT) and quantitative real-time polymerase chain reaction (PCR) were carried out as described previously[10]. Each sample was examined in triplicate and normalized to TATA-binding protein (*TBP*) messenger ribonucleic acid expression as an internal control. The primer sequences were the same as in our previous report[10]. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

### Bromodeoxyuridine incorporation assay

To evaluate the proliferation of ALVA-control or ALVA-41-GLP-1R cells with or without Forskolin (#F6886; Sigma-Aldrich, Tokyo, Japan), bromodeoxyuridine assay was carried out using a Cell Proliferation ELISA kit (1647229; Roche Applied Science, Mannheim, Germany), as described previously[10,11].

### Apoptosis assay

To detect apoptotic cells, terminal deoxynucleotidyl transferase dUTP nick-end labeling staining was demonstrated using the DeadEnd Fluorometric TUNEL System (Promega, Tokyo, Japan), according to the company's protocol, as previously described[10].

### Measurement of cyclic adenosine monophosphate concentration

Measurement of the cyclic adenosine monophosphate (cAMP) concentration was performed as described previously[10], using a cAMP Enzyme Immunoassay Kit (#501040; Cayman Chemical, Ann Arbor, MI, USA), according to the company's instructions.

### Cell cycle analysis by flow cytometry

Cell cycle analysis by flow cytometry was carried out as reported previously[6]. Briefly, ALVA-41 cells were seeded in 60-mm dishes at $1 \times 10^5$ cells/mL. Cells were cultured with 10 nmol/L Ex-4 or phosphate-buffered saline for 48 h and reached 60–70% confluency. Cell cycle analysis was carried out using a Cycletest™ Plus DNA reagent kit (BD Biosciences Franklin Lakes, NJ, USA), following the manufacturer's instructions, and BD FACSVerse (BD Biosciences). Flow cytometry data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

### Western blotting analysis

Western blotting was carried out as described previously[6,10]. The following primary antibodies were used: phospho-ERK (Thr-202/Tyr-204; #9101, Cell Signaling, Danvers, MA, USA),

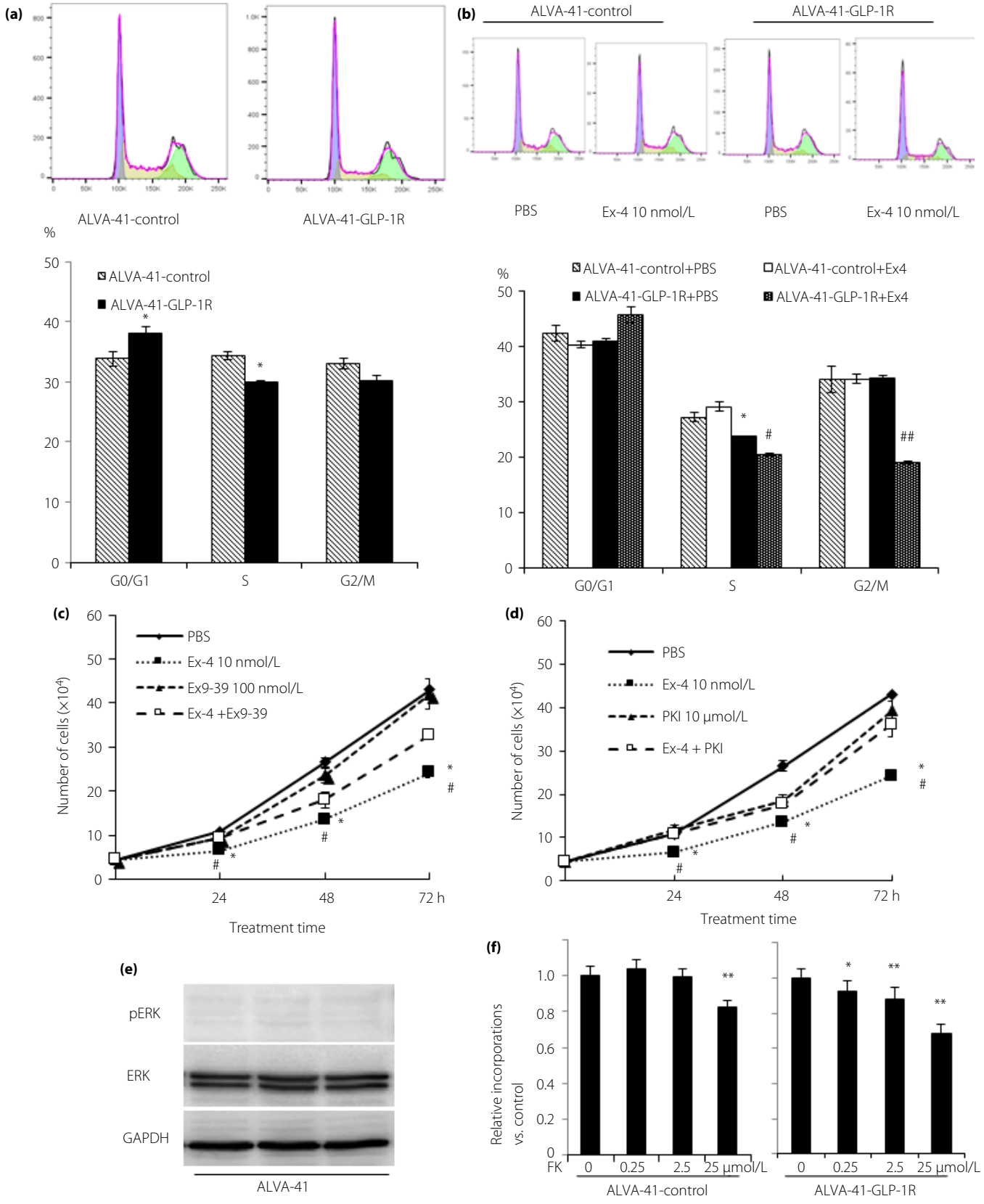


**Figure 3** | Cell cycle distribution and signal transduction. (a, b) Flow cytometric analysis was carried out to determine the cell cycle distribution of ALVA-41-control and glucagon-like peptide-1 receptor (GLP-1R) was overexpressed in ALVA-41 cells using a lentiviral vector (ALVA-41-GLP-1R) cells with or without Ex-4. Data are represented as the ratios of cells distributed in each phase to the total cells. The unpaired *t*-test was carried out to calculate statistical significance. (a) $^{*}P < 0.05$ versus ALVA-41-control ($n = 3$). (b) $^{*}P < 0.05$ versus ALVA-41-control and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus ALVA-41-GLP-1R + phosphate-buffered saline (PBS; $n = 3$). (c,d). Growth curves of ALVA-41-GLP-1R cells with or without Ex-4, Ex9–39 or PKI. The unpaired *t*-test was carried out to calculate statistical significance for (c) $^{*}P < 0.05$ versus PBS and $^{\#}P < 0.05$ versus Ex-4 + Ex9–39 ($n = 3$), and (d) $^{*}P < 0.05$ versus PBS and $^{\#}P < 0.05$ versus Ex-4 + PKI ($n = 3$). (e) Western blotting of phospho-ERK, ERK and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was carried out in three independent ALVA-41 cell lysate samples. (f) Bromodeoxyuridine assays were carried out to measure deoxyribonucleic acid synthesis in ALVA-41-control and ALVA-41-GLP-1R cells treated with or without forskolin (FK) for 24 h. Data are expressed as relative absorbance compared with 0 μmol/L forskolin in ALVA41-control or ALVA-41-GLP-1R cells. One-way ANOVA with a post-hoc Dunnett's test was carried out to calculate statistical significance. $^{*}P < 0.05$, $^{**}P < 0.01$ versus EtOH (0 μmol/L; $n = 5$). ERK, extracellular signal-related kinase; pERK, phosphorylated extracellular signal-related kinase.

ERK (#9102, Cell Signaling), cyclin D1 (#2978, Cell Signaling), phospho-Rb (Ser807/811; #8516, Cell Signaling), p27Kip1 (#3686; Cell Signaling) and glyceraldehyde 3-phosphate dehydrogenase (sc-20357; Santa Cruz Biotechnology, Dallas, TX, USA).

### Statistical analysis

The unpaired *t*-test or one-way ANOVA was carried out for statistical analysis as appropriate. *P*-values of <0.05 were considered as statistically significant. Results are expressed as the mean ± standard error of the mean.

## RESULTS

### GLP-1R expression in human prostate cancer is inversely associated with cancer progression

As we reported previously[10], GLP-1R is observed in prostate cancer tissue in non-diabetic individuals and colocalizes with P504S, a prostate cancer marker. Interestingly, as shown in Figure 1a, expression levels of GLP-1R were decreased in advanced prostate cancer cases categorized by Gleason score[13]. When the 30 patients were divided into three groups according to Gleason score, expression of GLP-1R in prostate cancer was significantly decreased in advanced prostate cancer patients with high Gleason scores compared with early-stage prostate cancer patients (Figure 1b).

### Forced expression of GLP-1R attenuates prostate cancer cell proliferation

To elucidate the effect of GLP-1R expression on prostate cancer, we overexpressed GLP-1R in prostate cancer cells. In our previous report[10], endogenous GLP-1R expression was negligibly detected in ALVA-41 cells. In the present study, we overexpressed human GLP-1R in ALVA-41 cells using a lentiviral vector. As shown in Figure 1c, *GLP1R* gene expression was abundantly detected in ALVA-41 cells transfected with the lentiviral vector carrying the human *GLP1R* gene (ALVA-41-GLP-1R cells) compared with LNCaP cells that express endogenous GLP-1R. However, GLP-1R expression was not detected in ALVA-41 cells transfected with the empty lentiviral vector (ALVA-41-control cells). Furthermore, immunohistochemistry of GLP-1R confirmed significant membranous GLP-1R protein expression in ALVA-41-GLP-1R cells (Figure 1d). The functional effectiveness of overexpressed GLP-1R was demonstrated by intracellular cAMP induction in ALVA-41-GLP-1R cells stimulated with Ex-4 (Figure 1e).

We next examined the anti-proliferative effect of GLP-1R in ALVA-41 cells. As shown in Figure 2a, the number of ALVA-41-GLP-1R cells was slightly, but significantly, reduced compared with ALVA-41-control cells without GLP-1R agonist treatment. In addition, Ex-4 decreased the number of ALVA-41-GLP-1R cells in a dose-dependent manner, as shown by the growth curve in Figure 2b. However, ALVA-41-control cells did not respond to Ex-4 (Figure 2c). Consistent with the growth curve data, bromodeoxyuridine incorporation assays showed that the proliferation of ALVA-41-GLP-1R cells was significantly decreased compared with that of ALVA-41-control cells (Figure 2d). In addition, Ex-4 attenuated ALVA-41-GLP-1R cell proliferation in a dose-dependent manner, but had no impact on ALVA-41-control cell proliferation (Figure 2e). Similar to our previous report using LNCaP cells[10], GLP-1R activation did not induce apoptosis of ALVA-41-GLP-1R cells (Figure 2f).

### Forced expression of GLP-1R attenuates cell cycle progression through inhibition of SKP2 and upregulation of p27Kip 1

We next examined the mechanism by which overexpressed GLP-1R attenuated ALVA-41 cell proliferation. First, we carried out cell cycle analysis by flow cytometry. As shown in Figure 3a, ALVA-41-GLP-1R cells in G0/G1 phase were increased and those in S phase were decreased compared with ALVA-41-control cells. Furthermore, Ex-4 treatment decreased not only S phase entry, but also G2/M phase transition of ALVA-41-GLP-1R cells (Figure 3b). Consistent with the apoptosis assay (Figure 2f), the sub-G1 fraction was not observed after Ex-4 treatment, further supporting suppression of apoptosis. Notably, significantly increased G0/G1 cells were observed in ALVA-41-GLP-1R cells compared with ALVA-41-control cells in Figure 3a, but not in Figure 3b. This might be an experimental limitation, because the experiment shown in Figure 3b involved a 12-h longer incubation time. We found that exendin (9–39), a GLP-1R antagonist (Figure 3c) and inhibitor of protein kinase A (Figure 3d), significantly counteracted Ex-4-induced

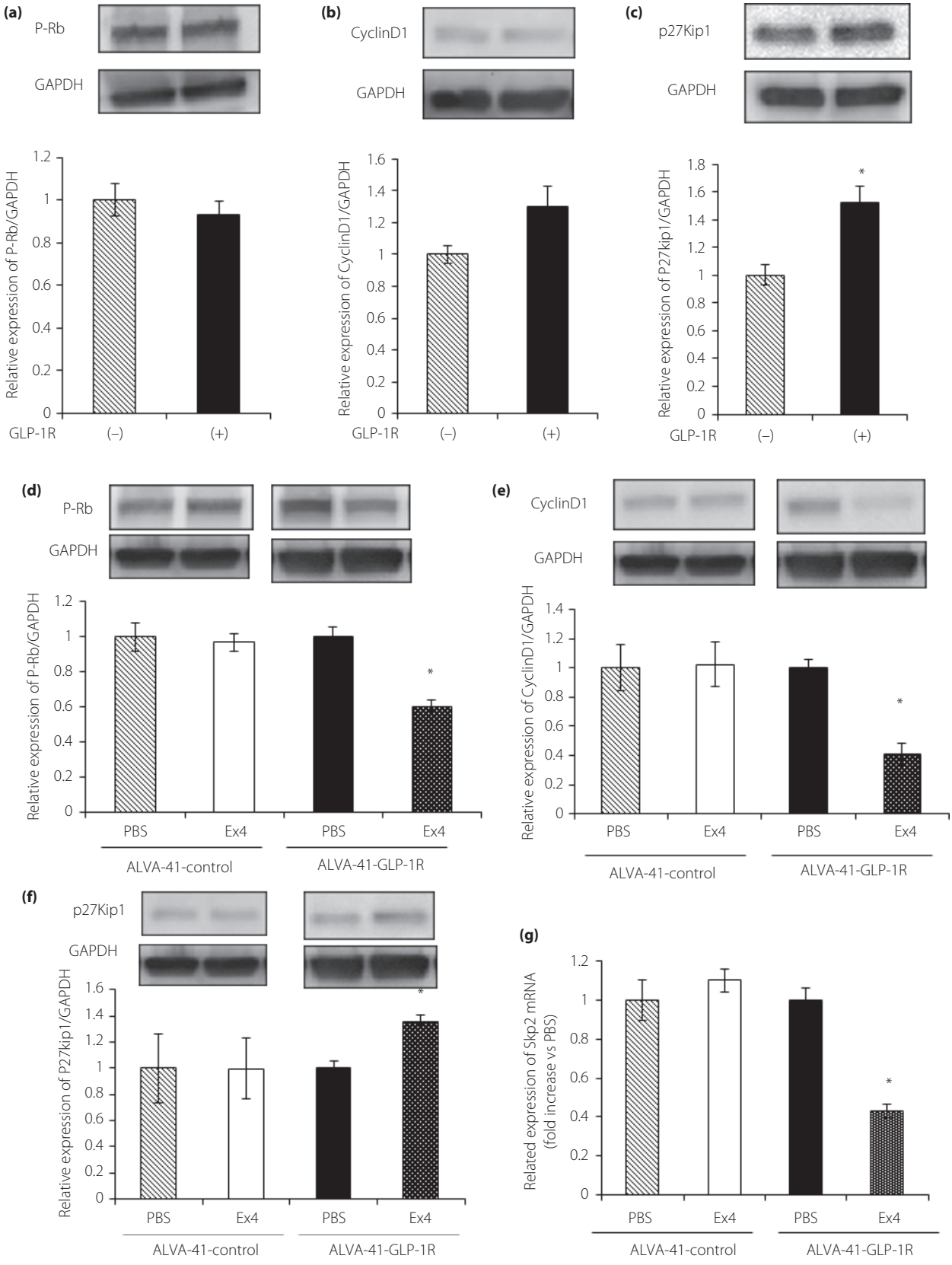


**Figure 4** | Expression of cell cycle regulators in glucagon-like peptide-1 receptor (GLP-1R) was overexpressed in ALVA-41 cells using a lentiviral vector (ALVA-41-GLP-1R) cells. Western blotting of (a,d) phosphorylated Rb, (b,e) cyclin D1 and (c,f) p27Kip 1 was carried out in ALVA-41-control and ALVA-41-GLP-1R cells with or without 10 nmol/L Ex-4 for 24 h. Densitometry was carried out by normalization to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data are represented as (a–c) relative expression to ALVA-41-control cells or (d–g) cells treated with phosphate-buffered saline (PBS). Quantitative real-time reverse transcription polymerase chain reaction of *SKP2* was carried out in ALVA-41-control and ALVA-41-GLP-1R cells with or without 10 nmol/L Ex-4 for 24 h. The unpaired *t*-test was carried out to calculate statistical significance. (c) $*P < 0.05$ versus ALVA-41-control and (d–g) $*P < 0.05$ versus ALVA-41-GLP-1R treated with PBS.

attenuation of cell proliferation, indicating that Ex-4 inhibited cell proliferation through the activation of GLP-1R and cAMP-protein kinase A signaling, a canonical pathway of GLP-1R. In our previous report using LNCaP cells, Ex-4 attenuated cell proliferation through inhibition of ERK[10]. However, ERK was not activated in ALVA-41 cells (Figure 3e). To confirm the anti-proliferative effect of intracellular cAMP induced by GLP-1R, we carried out bromodeoxyuridine assay with forskolin, which is a ubiquitous activator of eukaryotic adenylyl cyclase, to increase the cAMP level. As shown in Figure 3f, forskolin significantly decreased cell proliferation in ALVA-41 cells and further reduction of cell proliferation was observed in ALVA-41-GLP-1R cells.

Because G0/1 arrest was induced by GLP-1R activation in ALVA-41 cells, we carried out further experiments focusing on cell cycle regulators. Western blotting showed no significant differences in Rb protein phosphorylation and cyclin D1 expression between ALVA-41-GLP-1R and ALVA-41-control cells without Ex-4 treatment (Figure 4a,b). However, p27Kip1, a negative regulator of the G0/1-to-S phase transition, was significantly increased in ALVA-41-GLP-1R cells compared with ALVA-41-control cells (Figure 4c). Furthermore, Ex-4 treatment significantly decreased Rb phosphorylation (Figure 4d) and cyclin D1 expression (Figure 4e), and significantly increased p27Kip1 expression (Figure 4f) in ALVA-41-GLP-1R cells, but not in ALVA-41-control cells. Because p21Kip1 protein levels are post-translationally regulated by SKP2 ubiquitin ligase, we next examined SKP2 messenger ribonucleic acid expression by quantitative RT–PCR. As shown in Figure 4g, *SKP2* gene expression was decreased significantly by Ex-4 in ALVA-41-GLP-1R cells, but not in ALVA-41-control cells.

### Forced expression of GLP-1R attenuates prostate cancer growth *in vivo* independent of glucose metabolism

To determine the anti-prostate cancer effect of overexpressed GLP-1R *in vivo*, we implanted ALVA-41-GLP-1R or ALVA-41-control cells, which stably express cytomegalovirus-luciferase, as reported previously[16], into athymic nude mice. Four weeks after subcutaneous implantation of ALVA-41 cells into the flank region of mice, tumor formation was visualized by *in vivo* imaging of the fluorescence intensity derived from cytomegalovirus-luciferase in ALVA-41 cells just before being euthanized (Figure 5a). The tumor growth measured by the fluorescence intensity of ALVA-41-GLP-1R cells was decreased compared with that of ALVA-41-control cells without Ex-4, but it was not statistically significant. However, Ex-4 treatment significantly reduced the tumor growth of ALVA-41-GLP-1R cells compared with that of ALVA-41-control cells without Ex-4 (Figure 5b). In resected tumors, the calculated tumor growth of ALVA-41-GLP-1R cells was attenuated, but it was not statistically significant (Figure 5c). However, the tumor weight of ALVA-41-GLP-1R cells was significantly decreased compared with that of ALVA-41-control cells without Ex-4, and Ex-4 treatment significantly decreased the tumor weight of ALVA-41-GLP-1R cells compared with that of ALVA-41-control cells treated with Ex-4 (Figure 5d). During the experimental period, serum glucose levels and bodyweights were not significantly different between the four groups (Figure 5e,f).

## DISCUSSION

In the present study, we showed that expression of GLP-1R in human prostate cancer cells was inversely associated with cancer progression, and that forced expression of GLP-1R inhibited prostate cancer cell proliferation *in vivo* and *in vitro* by attenuating cell cycle progression. Incretin therapies have recently emerged as major anti-diabetic agents worldwide[17] including in Japan[18]. Several advantages of incretin therapy, such as protection of pancreatic β-cells, possibility of weight loss and fewer hypoglycemic events, have been reported[19]. Furthermore, incretin therapy is one of the therapeutic options for type 2 diabetes mellitus, even in chronic renal failure[20]. In addition, recent large-scale randomized controlled trials have suggested that GLP-1R agonists significantly reduced cardiovascular events[21,22]. Following this evidence, the early use of GLP-1R agonists is recommended by the American Diabetes Association, especially for patients with established atherosclerotic cardiovascular and chronic kidney diseases[23]. However, the currently emerging consideration for incretin therapies should be their long-term guaranteed safety, including the cancer risk.

The mechanism-of-action of GLP-1 on cancer is still under elucidation, as we described in a previous review[24]. Although some data have shown a risk of carcinogenesis from GLP-1R agonist use, there is no evidence in randomized controlled trials that GLP-1R agonists increase cancer onset or death. Nevertheless, we have previously investigated the anti-cancer effects of a GLP-1R agonist in not only prostate cancer models[10,11], but also in breast cancer models[15]. These data suggest anti-cancer effects of GLP-1R agonists. Among the numerous cancers associated with DM and metabolic syndromes[25], the association between prostate cancer and DM is controversial, and some

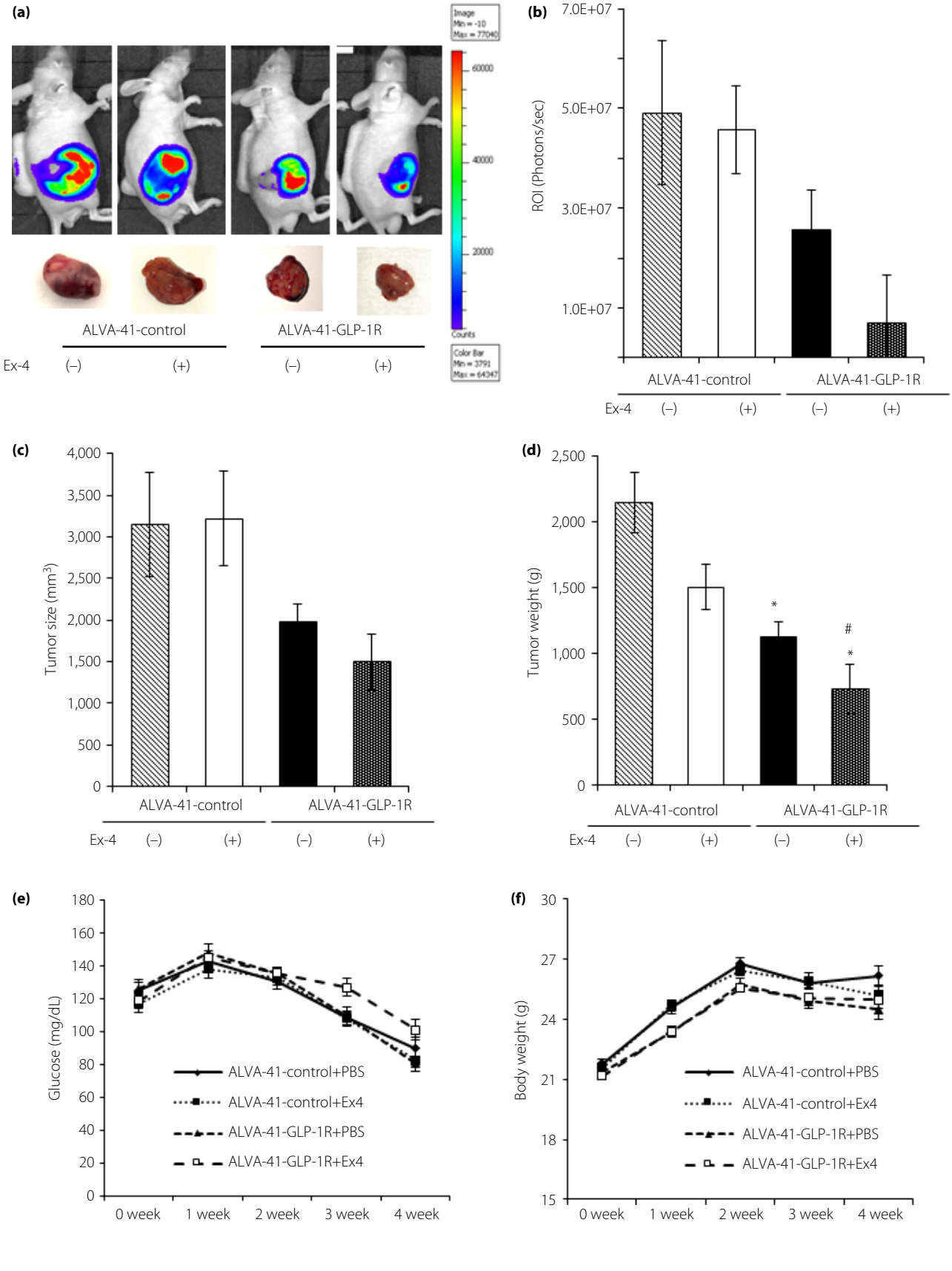


**Figure 5** | Forced expression of glucagon-like peptide-1 receptor (GLP-1R) attenuates prostate cancer growth *in vivo*. (a) ALVA-41-control or ALVA-41-GLP-1R cells stably transfected with the Luciferase gene were implanted into athymic nude mice with or without Ex-4 treatment. Tumor growth was visualized using an *in vivo* imaging system. (b) Quantification of fluorescence was determined in tumor cells. (c) Tumor volumes were calculated by the modified ellipsoid formula. (d) Tumor weight was measured by balance. (e) Plasma glucose and (f) bodyweight were measured during the experimental period. The unpaired *t*-test was carried out to calculate statistical significance: $^{*}P < 0.05$ versus ALVA-41-control + phosphate-buffered saline (PBS), $^{\#}P < 0.05$ versus ALVA-41-control + Ex-4.

data suggest that patients with type 2 diabetes mellitus have a lower risk of prostate cancer compared with non-diabetic individuals[26]. However, a higher incidence of prostate cancer has been observed in large-scale studies carried out in Western countries[12], as well as in Japan[9]. Furthermore, a higher body mass index and higher plasma C-peptide concentration increase prostate cancer mortality[27]. Previously, we showed that insulin-like growth factor-I and insulin increase prostate cancer cell proliferation *in vitro*[28]. These data suggest that caution is required regarding prostate cancer, especially in obese and insulin-resistant patients with type 2 diabetes mellitus. The expression level of GLP-1R was inversely associated with the Gleason score and prostate cancer advances (Figure 1a,b) in human prostate cancer. These data show that the activation of GLP-1R could be a marker of early-stage prostate cancer, and that GLP-1R agonist might be a therapeutic option for patients with type 2 diabetes mellitus complicated with early-stage prostate cancer based on our previous report[10] and the present study. In our earlier study, Ex-4 attenuated LNCaP cell proliferation by inhibiting ERK activation[10]. However, in ALVA-41 cells, ERK was not activated (Figure 3e). As explained in a previous review article[29], the ERK pathway is one of the most important growth signals in prostate cancer. However, ALVA-41 cells do not express ERK, probably because the ALVA-41 line is a cell line from bony metastasis from human prostate cancer, not primary prostate cancer[30], and some transformation might occur regarding growth signals. In addition, cAMP response element binding protein (CREB) is one of the critical transcriptional factors activated by ERK phosphorylation, and we previously showed that Ex-4 decreased CREB phosphorylation in vascular smooth muscle cells[6]. Although CREB was slightly detected in ALVA-41 cells, phosphorylation of CREB was not detected in ALVA-41 cells with or without GLP-1R expression and Ex-4 treatment (Figure S1). Furthermore, to confirm the anti-prostate cancer effect of GLP-1R, we carried out forced expression of GLP-1R in PC3 cells, a widely used prostate cancer cell line. As shown in Figure S2, Ex-4 attenuated cell proliferation in PC3 cells overexpressing GLP-1R. Interestingly, overexpression of GLP-1R and treatment with Ex-4 attenuated ALVA-41 cell proliferation by inhibiting cell cycle progression, which was independent of ERK activation, suggesting that GLP-1R activation attenuates cell proliferation by activating different signaling pathways depending on the cell type. In the present study, GLP-1R activation decreased SKP2 expression and subsequently increased p27Kip1 protein levels to induce G0/1 arrest and inhibit cell cycle progression. We reported a similar effect of Ex-4 in vascular smooth muscle cells (VSMCs)[6]. Ex-4 inhibited SKP2 expression and attenuated VSMC proliferation and neointima formation after vascular injury[6]. These data suggest that SKP2 might be a critical regulator of the anti-proliferative effect of GLP-1R activation in proliferating cells, such as cancer cells and VSMCs.

SKP2 is an F-box protein that regulates p27Kip 1 ubiquitination and degradation, functioning as an ubiquitin ligase[31]. Interestingly, an opposing interaction between GLP-1 action and SKP2 was reported by another study[32]. GLP-1 upregulates SKP2 expression and additionally downregulates p27Kip1 expression to accelerate cell proliferation through an insulin receptor substrate 2-dependent signal transduction in pancreatic β-cells[32]. The interaction between GLP-1 signaling and SKP2 is most likely influenced by cell proliferative activity and other growth signals. In the present study, the key mechanism by which Ex-4 attenuates ALVA-41 cell proliferation through GLP-1R activation could be upregulation of cAMP level, because forskolin also decreased ALVA-41 cell proliferation. In fact, cAMP activates SKP2 expression and attenuates cell proliferation in VSMCs[3,33]. SKP2 induction by cAMP increased by GLP-1R signaling could be one of the mechanisms by which GLP-1 attenuates prostate cancer growth, similar to ERK inhibition[10]. In addition, an association between GLP-1R and prostate cancer has not been investigated by genome-wide research. Although the relationships between variants of *TCF7L2* (transcription factor 7-like 2), one the most important transcription factors for *GLP-1R* expression, and some cancers, such as breast, colorectal and lung cancers, have been reported[34], further study is required.

Considering the primary action of GLP-1, GLP-1R agonists are anti-diabetic agents that induce anti-apoptotic action and cell proliferation of pancreatic β-cells through ERK, Akt and β-catenin activation[35]. However, the present data and our previous reports[10-11,15] suggested an anti-proliferative effect of GLP-1R agonist in cancer cells. These two actions appear to be opposite in effect. Koehler *et al.*[36] reported that the GLP-1R agonist Ex-4 does not modify cell growth and apoptosis in pancreatic cancer cells. GLP-1 action and downstream signals of GLP-1R might be different depending on the cell line and cancerous or not cancerous cells. Further research into the GLP-1R signal depending on cell background and in cross-talk with other signal transductions should be required.

In conclusion, we investigated an anti-prostate cancer effect by overexpressed GLP-1R activation *in vitro* and *in vivo*. The present study might facilitate establishing diabetes therapies to

prevent cancer, and GLP-1R activation might be an option for prostate cancer therapy.

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## DISCLOSURE

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1 |** Cyclic adenosine monophosphate response element-binding protein expression and phosphorylation in ALVA-41 cells.

**Figure S2 |** Overexpression of human glucagon-like peptide-1 receptor in PC3 cells.