Original Article

Combined treatment with glucagon-like peptide-1 receptor agonist exendin-4 and metformin attenuates breast cancer growth

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ABSTRACT

Cancer is a major cause of death in patients with diabetes. Incretin therapy has received much attention because of its tissue-protective effects. We have previously reported an anti-breast cancer effect of glucagon-like peptide-1 receptor agonist exendin-4 (Ex-4). An anti-cancer effect of metformin is well recognized. Therefore, we examined the effect of combined treatment with Ex-4 and metformin in breast cancer cells. In human breast cancer cell lines MCF-7, MDA-MB-231, and KPL-1, 0.1–10 mM metformin significantly reduced the cell number in growth curve analysis in a dose-dependent manner. Furthermore, combined treatment with 0.1 mM metformin and 10 nM Ex-4 additively attenuated the growth curve progression of breast cancer cells. In a bromodeoxyuridine (BrdU) assay, Ex-4 or metformin significantly decreased breast cancer cell proliferation and further reduction of BrdU incorporation was observed by combined treatment with Ex-4 and metformin, which suggested that Ex-4 and metformin additively

decreased DNA synthesis in breast cancer cells. Although apoptotic cells were not observed among Ex-4-treated breast cancer cells, apoptotic cells were clearly detected among metformin-treated breast cancer cells by apoptosis assays. Furthermore, metformin decreased BCL-2 expression in MCF-7 cells. *In vivo* experiments using a xenograft model showed that Ex-4 and metformin significantly decreased the breast tumor weight and Ki67-positive proliferative cancer cells, and metformin reduced the serum insulin level in mice. These data suggested that Ex-4 and metformin attenuated cell proliferation and metformin induced apoptosis in breast cancer cells. Combined treatment of Ex-4 and metformin may be an optional therapy to inhibit breast cancer progression.

Key words: GLP-1 receptor agonist, Metformin, Breast cancer, Cell proliferation, Apoptosis

INTRODUCTION

Recently, cancer has been emerging as a major cause of death in patients with diabetes mellitus (DM) [1]. Particularly in Japan, cancer is the current leading cause of death in patients with type 2 diabetes mellitus (T2DM). Accordingly, the Japan Diabetes Society and Japan Cancer Association have issued a warning for an increased cancer risk in patients with DM [2]. Additionally, cancer has become the leading cause of death in patients with DM in not only Japan, but also in England very recently [3]. Notably, T2DM and metabolic syndrome caused by obesity have been suggested to be associated with higher risks of many cancers [4]. This evidence suggests the need for a therapeutic strategy against T2DM, especially with obesity, which decreases both the blood glucose level and the risk and progression of cancer.

Incretin therapies, which include glucagon-like peptide-1 receptor (GLP-1R) agonists and dipeptidyl peptidase-4 (DPP-4) inhibitors, have become major treatment modalities for T2DM. Fortunately, incretin therapies decrease the blood glucose level with a lower risk of hypoglycemic attack and body weight gain compared with other anti-diabetic agents. Furthermore, tissue and organ-protective effects of incretin therapies, especially GLP-1R agonists, beyond a glucose-lowering effect have been reported [5]. Previously, we have reported a vascular protective effect of GLP-1R agonist exendin-4 (Ex-4) using atheroma formation in an atherosclerotic *apoE*$^{-/-}$ mouse model [6] and intimal thickness after vascular guide wire injury [7, 8]. Following our basic research studies, clinical randomized control trials revealed a vascular protective effect of GLP-1R agonists [9]. Furthermore, we have investigated an anti-cancer effect of GLP-1R agonists because cancer is the leading cause of death in Japanese patients with DM as described above. Previously, we have reported an anti-prostate cancer effect of Ex-4 by monotherapy [10], combined therapy with metformin [11], and a forced expression model of GLP-1R using a lentiviral vector [12]. Breast cancer is a major cancer in women,

particularly in western countries, which is accelerated in female patients who have DM. It has been reported that women with T2DM have a 1.2-fold higher risk of breast cancer than women without T2DM [13]. We also found that GLP-1R agonist attenuates breast cancer cell proliferation via activation of GLP-1R and subsequent inhibition of NF-κB activation [14]. Metformin is the first line anti-diabetic agent in western countries [15] and its anti-cancer effect is well recognized. Because our previous investigation suggested an anti-breast cancer effect of GLP-1R agonist Ex-4 [14], we examined the effect of combined treatment with Ex-4 and metformin in breast cancer cells.

MATERIALS AND METHODS

Cell culture and proliferation assay

MCF-7 and MDA-MB-231 human breast cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). KPL-1 human breast cancer cell line was kindly provided by Dr. Junichi Kurebayashi, Kawasaki Medical School [16]. All breast cancer cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cell proliferation assays were performed as described previously [10, 11, 12, 14] with minor modifications. Briefly, cells were seeded in 12-well culture plates and maintained in complete medium with or without 0.1–10 mM metformin and/or 10 nM Ex-4. Cell proliferation was analyzed after 0–3 days by cell counting using a hemocytometer.

Animals

Female athymic CAnN.Cg-*Foxn1nu*/CrlCrlj mice were purchased from Charles River Laboratories, Inc. (Kanagawa, Japan) and housed in a specific pathogen-free barrier facility at Fukuoka University. When the mice reached 6 weeks of age, they were treated with either saline (control, n = 10), Ex-4 (Sigma-Aldrich, Tokyo, Japan) at 300 pmol kg body weight−1 day−1 (n = 10) delivered by a mini osmotic pump (ALZEST, model 1004; DURECT, Cupertino, CA, USA), metformin (Wako Pure Chemical Industries, Ltd, Osaka, Japan) at 750 mg kg−1 day−1 by mixing with feed (n = 10), or combined Ex-4 and metformin (n = 10) as described previously [11]. Then, 1×10^6 MCF-7 cells (passage 4–8) were mixed with 250 μl Matrigel (Becton Dickinson Labware, Bedford, MA, USA) and implanted subcutaneously in the flank region. When the mice reached 14 weeks of age, blood samples were collected and the mice were euthanized. The tumor weight was measured by a balance and the tumor volume was calculated by a modified ellipsoid formula: length × width^2 × 0.52. Paraffin-embedded, formalin-fixed tumors were cut into 5-μm-thick sections and prepared for immunofluorescence staining. All procedures involving animals were reviewed and approved by the Institutional Animal Care Subcommittee at Fukuoka University Hospital. All institutional and national guidelines for the care and use of laboratory animals were followed.

Hematoxylin and eosin staining

The tissue sections were deparaffinized and rehydrated. The sections were stained in hematoxylin (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) for 5 minutes, and washed in running water for 15 minutes. Then, the sections were stained with eosin (Muto Pure Chemicals Co., Ltd.) for 3 minutes, dehydrated, and mounted by routine methods.

Immunofluorescence analysis

Paraffin-embedded sections were incubated with anti-Ki67 (ab66155; Abcam, UK) or anti-GLP-1R (NBP1-97308; Novus Biologicals, Littleton, CO) antibodies and then incubated with Alexa Fluor 488 goat anti-rabbit IgG (A-11008; Thermo Fisher Scientific, Rockford, IL, USA). Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and visualized by confocal microscopy.

Bromodeoxyuridine (BrdU) assay

To evaluate the proliferation of breast cancer cells, a BrdU incorporation assay was performed using a Cell Proliferation ELISA kit (1647229; Roche Applied Science, Mannheim, Germany) as described previously [10, 11, 12, 14].

Apoptosis assay

To label nuclei of apoptotic cells, 1.2×10^5 breast cancer cells were plated on glass coverslips in Lab-Tek Chamber Slides (177380; Nunc, Thermo Scientific, Waltham, MA, USA) and fixed in 4% paraformaldehyde for 25 min. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed using a DeadEnd Fluorometric TUNEL System (Promega, Madison, WI, USA) in accordance with the manufacturer's protocol. During the final 24 h, the cells were incubated with or without Ex-4 and metformin. Cells treated with 1 U/100 μl RQ1 RNase-Free DNase (M6101; Promega) for 10 min were used as a positive control.

Additionally, an Apoptosis/Necrosis Detection Kit (blue, green, red) (ab176749; Abcam) was used in accordance with the manufacturer's protocol. This kit distinguishes between viable, apoptotic, and necrotic cells by fluorescence microscopy.

Small interfering (si)RNA knockdown of *GLP-1R* expression and cell proliferation assay

To knockdown *GLP-1R* expression, we used Stealth RNAi Pre-Designed siRNA (Invitrogen, Carlsbad, CA, USA) that was designed to target human *GLP-1R* (HSS104179-81). Stealth RNAi Negative Control Duplexes (Invitrogen) were used as a negative control. For transfection, MCF-7 cells were seeded at 1.2×10^5 cells/well in 6-well plates and transfected with 10 nmol/l siRNA that targeted *GLP-1R* or negative control siRNA using MISSION siRNA Transfection Reagent (Sigma-Aldrich). Seventy-two hours after transfection, the cells were subjected to the cell proliferation assay. Briefly, cells were detached and reseeded in 24-well tissue culture plates in complete medium with or without 10 nM Ex-4 and 0.1 mM metformin. Seventy-two hours after treatment, the cells were collected and counted using a hemocytometer. siRNA knockdown efficiency was confirmed by immunocytochemical analysis of GLP-1R.

Western blot analysis

Western blotting was performed as described previously [10, 11, 12 14]. The following primary antibodies were used: anti-phospho-AMPKα (Thr172) (#2535; Cell Signaling Technology, Danvers, MA, USA), anti-AMPKα (#2532; Cell Signaling Technology), anti-GAPDH (sc-47724; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho-Akt (Thr-308) (#2965; Cell Signaling Technology), anti-phospho-Akt (Ser-473) (#4060; Cell Signaling Technology), anti-Akt (#4691; Cell Signaling Technology), anti-Caspase-9 (#9502;

Cell Signaling Technology), and anti-BCL-2 (#15071; Cell Signaling Technology). Protein expression was examined in MCF-7 cells incubated in medium with 10% FBS and stimulated with or without 10 nM Ex-4 and 0.1 mM metformin.

Insulin measurement

Insulin concentrations in mouse sera were measured using an Ultra Sensitive Mouse Insulin ELISA Kit (Morinaga Institute of Biological Science, Inc. Kanagawa, Japan) in accordance with the manufacturer's protocol as described previously [11].

Statistical analysis

One-way ANOVA and the unpaired *t*-test were performed for statistical analysis as appropriate. *P*-values of <0.05 were considered statistically significant. Results are expressed as the mean ± SEM.

RESULTS

Ex-4 and metformin decrease the breast cancer cell number in a growth curve

In our previous report, breast cancer cell proliferation was attenuated by Ex-4 in a dose-dependent manner [14]. In the present study, metformin also significantly decreased the number of breast cancer cells in a dose-dependent manner in MCF-7, MDA-MB-231, and KPL-1 cell lines (Figure 1a). We next examined whether combined therapy with Ex-4 and metformin attenuated breast cancer cell growth additively. As shown in Figure 1b, Ex-4 and metformin additively decreased the breast cancer cell number significantly in all three kinds of breast cancer cell lines.

Ex-4 and metformin decrease breast cancer cell proliferation and metformin induces apoptosis

We next examined the mechanism by which combined therapy with Ex-4 and metformin decreased the breast cancer cell number. BrdU assays revealed that both Ex-4 and metformin attenuated the proliferation of breast cancer cells significantly. Furthermore, combined therapy with Ex-4 and metformin additively decreased breast cancer cell proliferation (Figure 2a). In TUNEL assays, apoptotic breast cancer cells were observed after metformin treatment (Figure 2b), but not among Ex-4-treated cells, which was compatible with our previous report [14]. Because MCF-7 is the most popular breast cancer cell line, the following experiments were performed using MCF-7 cells. To confirm that metformin induced apoptosis of breast cancer cells, we demonstrated apoptotic and necrotic cell death induction using another assay system. As shown in Figure 2c, apoptotic cells were detected after treatment with metformin, but not Ex-4. Additionally, necrotic cells were not detected after metformin or Ex-4 treatments. To investigate the molecular target of metformin-induced apoptosis in breast cancer cells, we detected BCL-2 and Caspase-9 by western blotting. As shown in Figure 2d, metformin did not induce Caspase-9 cleavage. However, metformin, but not Ex-4, significantly decreased BCL-2 expression in MCF-7 cells (Figure 2e).

Ex-4 attenuates breast cancer cell proliferation by GLP-1R activation and metformin activates AMPK phosphorylation

Western botting showed that metformin, but not Ex-4, activated AMPK significantly (Figure 3a, b). In our previous report [14], we detected GLP-1R in breast cancer cell lines and revealed its expression in MCF-7 cells that were used in the present study. Immunofluorescence analysis of GLP-1R clearly confirmed its

expression in MCF-7 cells (Figure 3c). The GLP-1R expression level was not changed by Ex-4 and/or metformin treatments. To further examine the role of GLP-1R in combined treatment of breast cancer cells with Ex-4 and metformin, we knocked down *GLP-1R* in MCF-7 cells using siRNA. As shown in our previous report [14], the anti-proliferative effect of Ex-4 was cancelled by *siGLP-1R*, but metformin and combined treatment decreased the number of MCF-7 cells regardless of *GLP-1R* expression (Figure 3d). In all time points and treatments, *siGLP-1R cell* numbers were significantly lower than *siControl* cell numbers. To adjust the basal condition in knockdown cells, we calculated relative proliferation ratio compared with 0hr with *siControl* or *siGLP-1R* (Figure 3d). Similar to cell number counting results, the anti-proliferative effect of Ex-4 was cancelled by *siGLP-1R*, but not metformin and combined treatment and significant lower proliferating ratio was observed in *siGLP-1R* compared with siControl. Knockdown efficiency was confirmed by immunofluorescence analysis of GLP-1R (Figure 3e). We also examined Akt phosphorylation by western blotting, because Akt phosphorylation is one of the most important growth signals in breast cancer cells. As shown in Figure 3f and Figure 3g, Akt phosphorylation in serine, but not threonine, was significantly decreased by combined treatment.

Ex-4 and metformin decrease breast cancer growth *in vivo*

We performed *in vivo* experiments using a xenograft model [10, 11, 12, 14]. MCF-7 cells were implanted into athymic mice and treated with Ex-4, metformin, or both. As shown in Figure 4a. Although tumor volumes were smaller in Ex-4- and metformin-treated groups, significant reduction of the tumor volume was observed only in the metformin group (Figure 4b). However, measuring the tumor weigh demonstrated that combined therapy significantly decreased breast cancer growth (Figure 4c). Furthermore, immunofluorescence analysis

revealed that Ki67-positive proliferative cells were significantly decreased by treatment with Ex-4, metformin, and combined therapy (Figure 4d). During the study period, mouse body weight was unchanged among the four groups (Figure 5a) and the blood glucose level was decreased by combined therapy at 11 and 12 weeks (Figure 5b). The serum insulin level was also significantly decreased by metformin treatment (Figure 5c). Immunofluorescence analysis for GLP-1R revealed that Ex-4 and metformin increased GLP-1R expression in MCF-7 cells *in vivo* (Figure 5d). Furthermore, induction revel of GLP-1R was significantly higher in metformin treatment compared with Ex-4 (Figure 5e). In addition, HE staining of tumor section revealed that necrotic lesions of tumor were observed especially, in control group (Figure 5f).

DISCUSSION

In the present study, we found that combined therapy with GLP-1R agonist Ex-4 and metformin additively attenuated breast cancer growth by inhibiting cell proliferation and inducing apoptosis. Metformin is the most popular anti-diabetic agent worldwide and is recommended as the first line therapy for patients with T2DM in western countries [15]. The advantages of metformin are low risks of hypoglycemia and body weight gain, a high glucose-lowering effect, low cost, and beneficial in cardiovascular events [15]. Additionally, newly identified glucose-lowering mechanisms, such as glucose excretion into intestines [17], are surprisingly still being reported. Furthermore, an anti-cancer effect of metformin has been well recognized in both clinical trials and basic science [18]. The mechanism by which metformin attenuates cancer growth consists of indirect and direct effects. As indirect effects, metformin decreases serum insulin and insulin-like growth factor-1 by reduction of insulin resistance and induces apoptosis and cell cycle arrest in cancer cells as direct effects [18].

GLP-1R agonists have been emerging as one of the most popular anti-diabetic agents and they are recommended as the second line treatment following metformin for patients with T2DM, especially complicated with atherosclerotic and chronic kidney diseases [15]. However, the anti-cancer effect of GLP-1R agonists is unclear and remains under elucidation and controversy [19]. In fact, we have demonstrated an anti-prostate cancer effect of GLP-1 receptor agonist Ex-4 in basic experiments [10, 11, 12]. Following our report, a randomized control trial using GLP-1 receptor agonist liraglutide demonstrated that the GLP-1 receptor agonist decreased the prostate cancer risk in patients with T2DM [20]. Breast cancer is a major cancer in not only western countries, but also in Japan, and T2DM contributes to the risk of breast cancer [21]. We have previously found that GLP-1R agonist Ex-4 attenuates breast cancer growth through NF-κB inhibition both *in vivo* and *in vitro* [14]. Following our report, other groups have also reported that GLP-1R agonists attenuate breast cancer cell proliferation by modulating expression of adipokines [22] and microRNAs [23]. Interestingly, a recent clinical study reported that T2DM patients treated with DPP-4 inhibitors have decreased risks of breast cancer compared with treatment by metformin [24] or patients treated without DPP-4 inhibitors [25]. This might support our experimental data as an anti-breast cancer effect of incretin therapy. However, recent basic experimental data addressed warn that DPP-4 inhibitors accelerated epithelial-to-mesenchymal transition via C-X-C motif chemokine 12, one of the targets of DPP-4, activation in breast cancer cell [26]. In this aspect, GLP-1R agonist cold work better for anti-breast cancer effect among incretin therapy. However, at present, there is no evidence that suggests an anti-breast cancer effect of GLP-1R agonists. Further clinical elucidation focusing on GLP-1R agonist and breast cancer is required.

In the present study, we investigated additive anti-breast cancer effects of Ex-4 and metformin. Because an anti-breast cancer effect was observed in both estrogen-dependent and -independent cell lines, this effect may

be independent of hormonal activity, which is compatible with our previous reports [10, 14]. Our previous report showed that GLP-1R agonist Ex-4 attenuates breast cancer cell proliferation by activating GLP-1R [14]. In the present study, knocking down GLP-1R also canceled the anti-breast cancer effect of Ex-4, but not metformin, which suggested that the effect of Ex-4 was dependent on GLP-1R, whereas metformin was not dependent. The present data suggested the importance of GLP-1R expression in anti-proliferative effect of Ex-4 in breast cancer cells. Surprisingly, GLP-1R knocking down decreased cell number and proliferation ratio generally, compared with *siControl* cells. One possibility is that receptor knocking down gave much more damage to breast cancer cells compared with control. Another possibility is that endogenous GLP-1R expression may contribute to cell proliferation and cell viability. Actually, we observed reduced cell number by *siGLP-1R* in our previous report using prostate cancer cell [10]. These data suggested that endogenous GLP-1R expression may stimulate cell proliferation in cancer cell, whereas GLP-1R ligand attenuates cancer cell proliferation. A remarkable reduction in cell number induced by metformin was observed in *siGLP-1R* compared with *siControl*. These data suggest that a common mechanism exerts the anti-proliferative effect of both metformin and the GLP-1R agonist. A study has reported that not only metformin but also GLP-1 activates AMPK in the brain and liver [27]. Additionally, there is the possibility of another common mechanism by which metformin and the GLP-1R agonist attenuate cancer cell proliferation. To investigate detailed interaction between Ex-4 and metformin, further experiment should be required. In the present study, metformin, but not Ex-4, activated AMPK and induced apoptosis. AMPK is one of the most important target molecules in not only energy metabolism [27], but also anti-cancer effects [28]. GLP-1 also activates AMPK [27], but AMPK activation by Ex-4 was not observed in the present study or our previous study using a prostate cancer model [11]. Most likely, GLP-1 might not be able to activate AMPK in cancer cells. In the present

study, metformin decreased expression of anti-apoptotic protein BCL-2 and subsequently induced apoptosis. Another study has also reported that metformin induces apoptosis by reducing BCL-2 expression in breast cancer cells [29]. Further study is expected to investigate the anti-cancer effect of metformin mediated via mitochondrial biogenesis. *In vivo*, we did not observe consistent results of the tumor size. The tumor volume was the smallest in the metformin group, but the weight was the smallest in the combined treatment group. However, we believe that weight is more reliable than volume, because the modified ellipsoid formula is a virtual calculation that depends on an ellipsoid shape of the tumor. Cancers are not always an ellipsoid shape. In addition, we observed necrotic lesion in tumor section by HE staining. This could be one of the reasons why tumor volume data could not be similar to tumor weight data. *In vivo*, we detected an anti-proliferative effect, but not apoptosis, AMPK activation, and BCL-2 reduction, because of the sample volume limitation. Therefore, further elucidation is required. Body weight was the same among the four groups, which indicated that the mice were not in cachexia during the study period. To focus on anti-cancer effects independent of lowering glucose, we used non-diabetic mice. Their blood glucose level was normal and did not affect anti-breast cancer effects. Reduction of the serum insulin revel is an indirect anti-cancer effect of metformin [18], but not Ex-4 that is a glucose-dependent insulin secretagogue *in vivo*. Similar to our previous report [11], metformin increased GLP-1R expression in breast cancer *in vivo*. This could be the mechanism by which Ex-4 and metformin attenuated breast cancer growth in synergistic effect. In the present study, Ex-4 also increased GLP-1R expression, but it was lower than metformin. In addition to our present and previous data, anti-cancer effects are exerted by the interaction between incretin therapy and metformin via epithelial-to-mesenchymal transition [30]. Combined therapy of T2DM and cancer biology may be an exciting task for diabetology in the near future.

Combined therapy with a GLP-1 receptor agonist and metformin is frequently administered to patients with T2DM for blood glucose control because it may be a very effective combination compared with other combinations [31]. However, the anti-cancer effect of this combined therapy is undergoing elucidation. In the J-DOIT3 study, multifactorial intensive intervention decreased cardiovascular events in patients with T2DM [32]. However, cancer death was not decreased by intensive therapy in J-DOIT3, although cardiovascular death was negligible during the study period [32]. This suggest that a diabetes treatment strategy should be considered to avoid cancer death. Hopefully, the present study may facilitate this in the future.

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AUTHOR CONTRIBUTIONS

Y.T., C.I., T.K., and Y.H. performed experiments and data analysis. T.H., T.S., T.Y., and D.K. reviewed the manuscript. T.N. wrote the manuscript and conceived the research hypothesis and design.

COMPLIANCE WITH ETHICAL STANDARDS

T.N. received lecture fees from Sumitomo Dainippon Pharma, Ono Pharmaceutical, Nippon Boehringer Ingelheim, and Nihon Eli Lilly. D.K. received lecture fees from Ono Pharmaceutical, Novo Nordisc Pharma, MSD, Sanofi K.K., and Takeda Pharmaceutical, and research grants from Nippon Boehringer Ingelheim, Takeda Pharmaceutical, Sumitomo Dainippon Pharma, and Ono Pharmaceutical. The other authors declare that they have no conflicts of interest.

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FIGURE LEGENDS

Figure 1. Ex-4 and metformin decrease the breast cancer cell number in a growth curve

(a) MCF-7, MDA-MB-231, and KPL-1 cells were maintained in medium supplemented with 10% fetal bovine serum (FBS) and phosphate buffered saline (PBS) or metformin (0.1–10 mM). After 0, 24, 48, and 72 h, the cells were harvested and cell proliferation was analyzed by cell counting using a hemocytometer. Control (PBS), black circles with solid line; metformin (0.1 mM), black squares with dotted line; metformin (1 mM), white circles with solid line; metformin (10 mM), white squares with dotted line. One-way ANOVA was performed to calculate statistical significance. $*P < 0.05$, $**P < 0.01$ vs. control (n = 3). (b) MCF-7, MDA-MB-231, and KPL-1 cells were maintained in medium supplemented with 10% FBS and PBS or metformin (0.1 mM) and/or Ex-4 (10 nM). After 0, 24, 48, and 72 h, the cells were harvested and cell proliferation was analyzed by cell counting using a hemocytometer. Control (PBS), black circles with solid line; Ex-4 (10 nM), black squares with dotted line; metformin (0.1 mM), white circles with solid line; Ex-4 and metformin, white

squares with dotted line. One-way ANOVA was performed to calculate statistical significance. *$P < 0.05$, **$P < 0.01$ vs. control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. Ex-4, $^{\dagger}P < 0.05$ vs. metformin (n = 3).

Figure 2. Ex-4 and metformin decrease breast cancer cell proliferation and metformin induces apoptosis.

(a) MCF-7, MDA-MB-231, and KPL-1 cells were seeded at a density of 5000 cells/well in 96-well plates in medium supplemented with 10% FBS and incubated with PBS or metformin (0.1 mM) and/or Ex-4 (10 nM) for 24 h. A BrdU solution was added during the last 2 h and the cells were harvested to measure DNA synthesis using a microplate reader at 450–620 nm. Mean data are expressed as the relative ratio to the proliferation of control (untreated) cells. One-way ANOVA was performed to calculate statistical significance. *$P < 0.05$, **$P < 0.01$ vs. control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. Ex-4, $^{\dagger\dagger}P < 0.01$ vs. metformin (n = 3). (b) MCF-7, MDA-MB-231, and KPL-1 cells were seeded on glass coverslips in 2-well chamber slides. After incubation with Ex-4 (10 nM) and/or metformin (0.1 mM) or 1 U/100 μl RQ1 DNase for 10 min, apoptotic cells were detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Images are representative of three independent experiments. (c) MCF-7 cells were seeded on glass coverslips in 24-well chamber slides. After incubation with Ex-4 (10 nM) and/or metformin (0.1 mM) for 24 h, apoptotic and necrotic cells were detected by an Apoptosis Necrosis Assay kit. MCF-7 cells were stimulated with the control (PBS) or Ex-4 (10 nM) and/or metformin (0.1 mM) for 24 h. (d) Western blotting of Caspase-9 and GAPDH. As a positive control, MCF-7 cells were treated with 1μM staurosporine or DMSO for 3 hours. (e) Western blotting of BCL-2 and GAPDH. Densitometry was conducted by comparison of BCL-2/GAPDH. Data are

represented as relative expression to the control (PBS). The one-way unpaired *t*-test was performed to calculate statistical significance. $^{**}P < 0.01$ vs. control, $^{\#}P < 0.05$ vs. Ex-4 (n=3).

Figure 3. Ex-4 attenuates breast cancer cell proliferation via GLP-1R activation and metformin activates AMPK phosphorylation.

(a) MCF-7 cells were stimulated with the control (PBS) or AICAR, positive control, or Ex-4 (10 nM) and/or metformin (0.1 mM) for 24 h. Western blotting of Phospho-AMPK, AMPK, and GAPDH is shown. (b) Densitometry was conducted by comparison of Phospho-AMPK/AMPK. Data are represented as relative expression to the control (PBS). One-way ANOVA was performed to calculate statistical significance. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. Ex-4 (n=3). (c) MCF-7 cells were treated with the control (PBS) or Ex-4 (10 nM) and/or metformin (0.1 mM) for 24 h. Subsequently, immunofluorescence analysis was performed to examine GLP-1R expression in breast cancer cell lines. All samples were counterstained with DAPI (magnification, ×400). GLP-1R-positive cells were counted and normalized against DAPI staining in four individual fields of view. Unpaired *t*-tests were performed to calculate statistical significance. (d) MCF-7 cells were transfected with either negative control duplexes or small interfering (si)RNA that targeted *Control* (white bar) or *GLP-1R* (black bar) and maintained in medium with 10% FBS and saline, 10 nM Ex-4 and/or 0.1 mM metformin. After 0 h and 72 h, the cells were harvested and cell proliferation was analyzed by cell counting using a hemocytometer. Relative proliferation ratio was calculated comparing with 0hr cell number in *Control* (white bar) or *GLP-1R* (black bar). Unpaired *t*-tests were performed to calculate statistical significance ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. control with 72 h FBS, $^{\#\#}P < 0.01$ vs.*siControl*.) (n = 5). (e) immunofluorescence analysis of GLP-1R was performed after transfection of

MCF-7 cells with vectors that contained a control siRNA (*si control*) or siRNA that targeted *GLP-1R* (*si GLP-1R*). (f) MCF-7 cells maintained in medium with 10% FBS were stimulated with the control (PBS) or 10 nM Ex-4 and/or 0.1 mM metformin for 24 h. Cell lysates were prepared and subjected to western blotting to assess phosphorylated Akt (Thr-308)/Akt (Ser-473)/Akt/GAPDH. (g) Densitometry was conducted by comparison of Phospho-Akt (Ser-473)/Akt. Data are represented as relative expression to the control (PBS). One-way ANOVA was performed to calculate statistical significance. $^{*}P < 0.05$ vs. control, $^{\#\#}P < 0.01$ vs. Ex-4, $^{\dagger}P < 0.05$ vs. metformin (n=3).

Figure 4. Ex-4 and metformin decrease breast cancer growth *in vivo*.

(a) Athymic CAnN.Cg-*Foxn1nu*/CrlCrlj mice (6 weeks old) were implanted with 1×10^6 MCF-7 cells (passage 4–8) and treated with either saline (control, n = 10), Ex-4 at 300 pmol kg body weight−1 day−1 (n = 10) delivered by a mini osmotic pump, metformin at 750 mg kg−1 day−1 by mixing with feed (n = 10), or combined Ex–4 and metformin (n = 10) for 8 weeks. A representative tumor is shown. Tumors were resected and the tumor volume was calculated by the modified ellipsoid formula (b) and tumor weight was measured using a balance (c). One-way ANOVA was performed to calculate statistical significance. $^{*}P < 0.05$ vs. control. (d) Sections (5 μm thick) were subjected to immunofluorescence analysis of Ki67 and counterstained with 4′,6-diamidino-2-phenylindole (DAPI; magnification, ×400). Ki67-positive cells were quantified by analyzing the fraction of stained cells in the tumor relative to the total number of nuclei. Values are expressed as the percentage of positive cells. One-way ANOVA was performed to calculate statistical significance. $^{*}P < 0.05$ vs. control.

Figure 5. Metformin decreases the serum insulin level.

Mouse body weight (a) and casual plasma glucose in *ad libitum*-fed mice (b) were measured during the study period. (c) Plasma insulin was measured when the mice reached 14 weeks of age. One-way ANOVA was performed to calculate statistical significance. $*P < 0.05$, $**P < 0.01$ vs. control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. Ex-4 (n=10). (d) Sections (5 μm thick) were subjected to immunofluorescence analysis of GLP-1R and counterstained with 4′,6-diamidino-2-phenylindole (DAPI; magnification, ×400). (e) GLP-1R positive cells were quantified by analyzing the fraction of stained cells in the tumor relative to the total number of nuclei. Values are expressed as the percentage of positive cells. Unpaired *t* test was performed to calculate statistical significance. $*P < 0.05$, $**P < 0.01$ vs. control, $^{\#}P < 0.05$ vs. Ex-4 (n=10).

https://link.springer.com/article/10.1007/s13340-021-00560-z

Figure 1

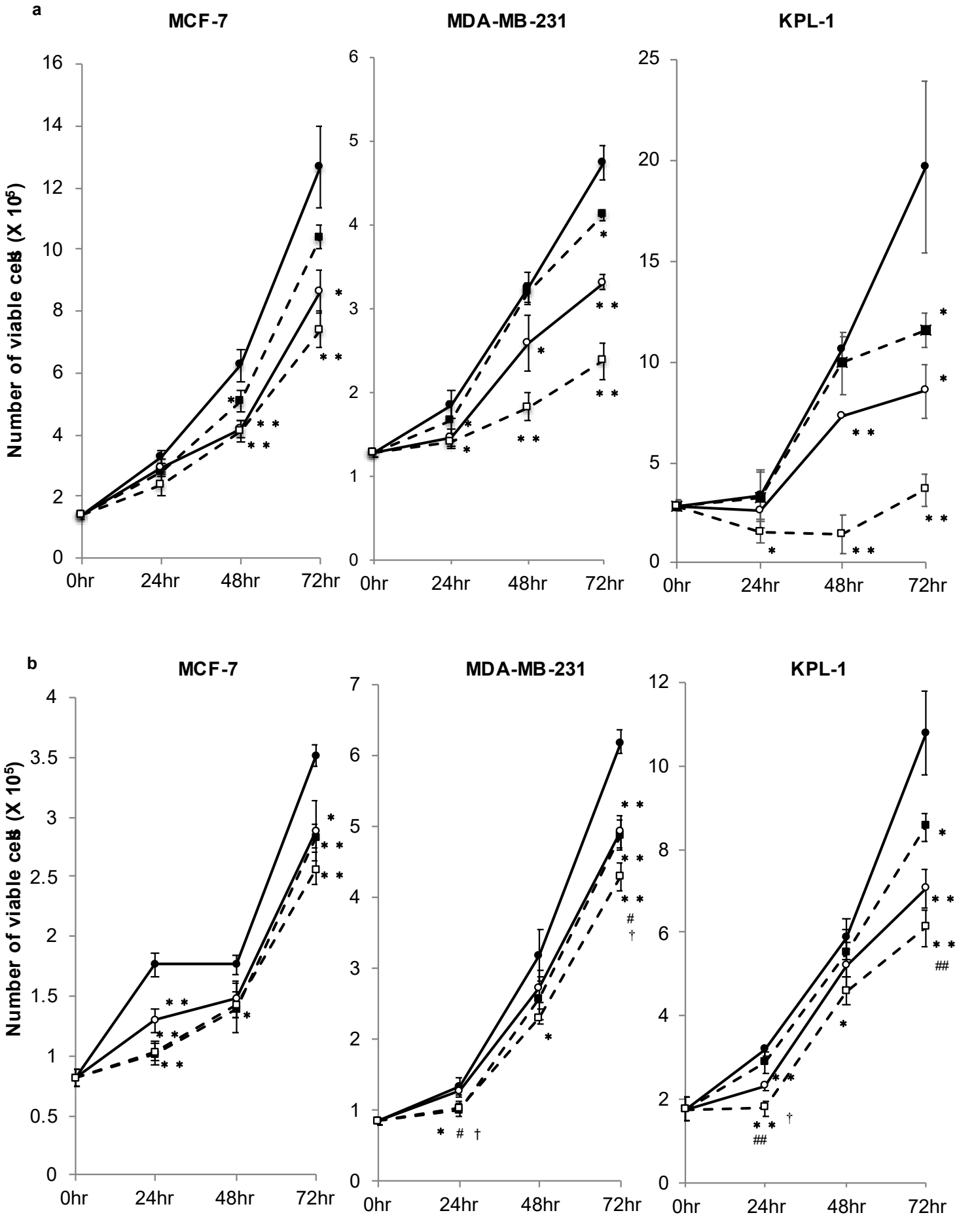

a
MCF-7
MDA-MB-231
KPL-1
Number of viable cells (X 10^5)
0hr
24hr
48hr
72hr
b
MCF-7
MDA-MB-231
KPL-1
Number of viable cells (X 10^5)
0hr
24hr
48hr
72hr

Figure 2

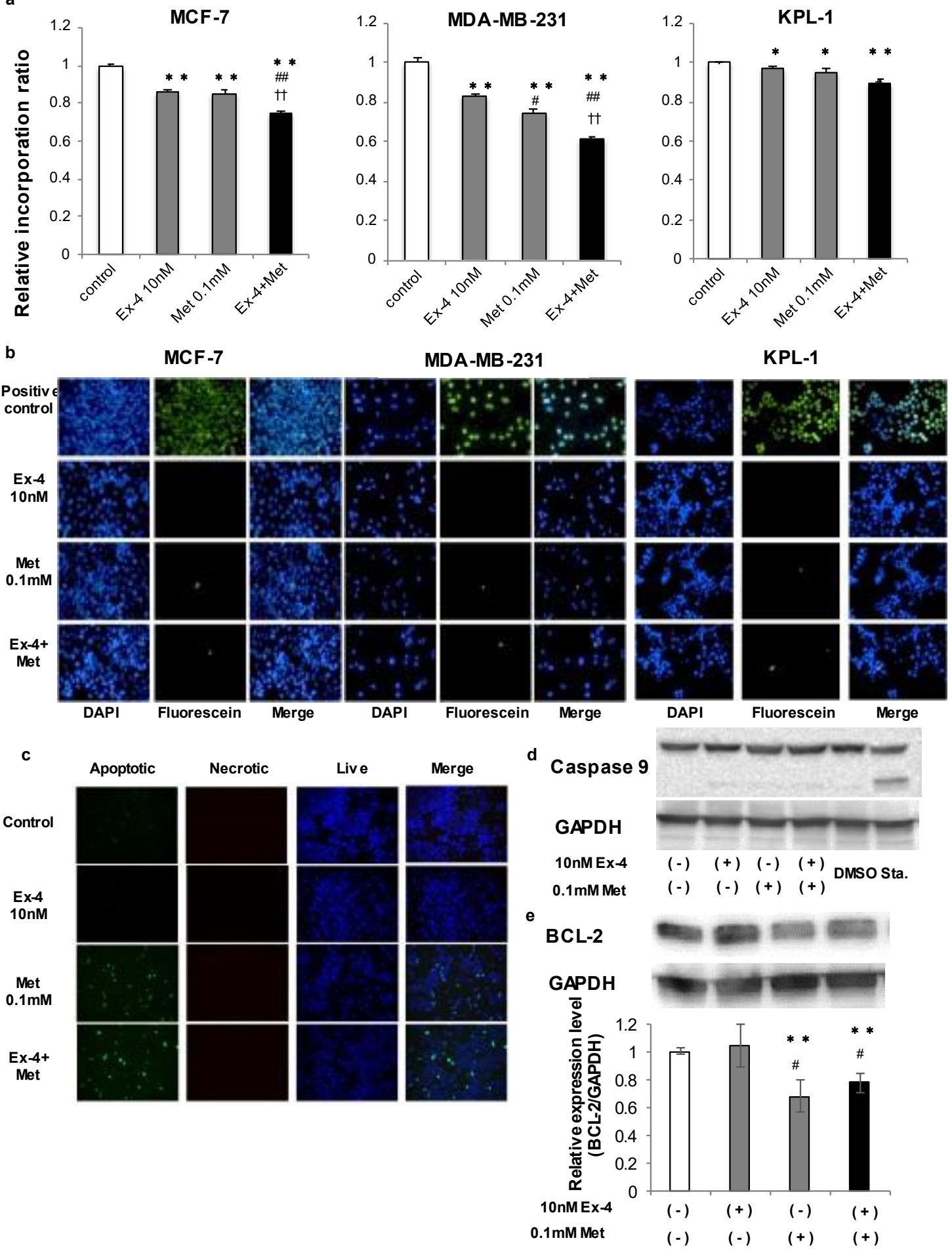

a
MCF-7
MDA-MB-231
KPL-1
Relative incorporation ratio
control
Ex-4 10nM
Met 0.1mM
Ex-4+Met
b
Positive control
Ex-4 10nM
Met 0.1mM
Ex-4+ Met
DAPI
Fluorescein
Merge
c
Apoptotic
Necrotic
Live
Merge
Control
d
Caspase 9
GAPDH
10nM Ex-4
0.1mM Met
DMSO Sta.
e
BCL-2
GAPDH
Relative expression level (BCL-2/GAPDH)

Figure 3

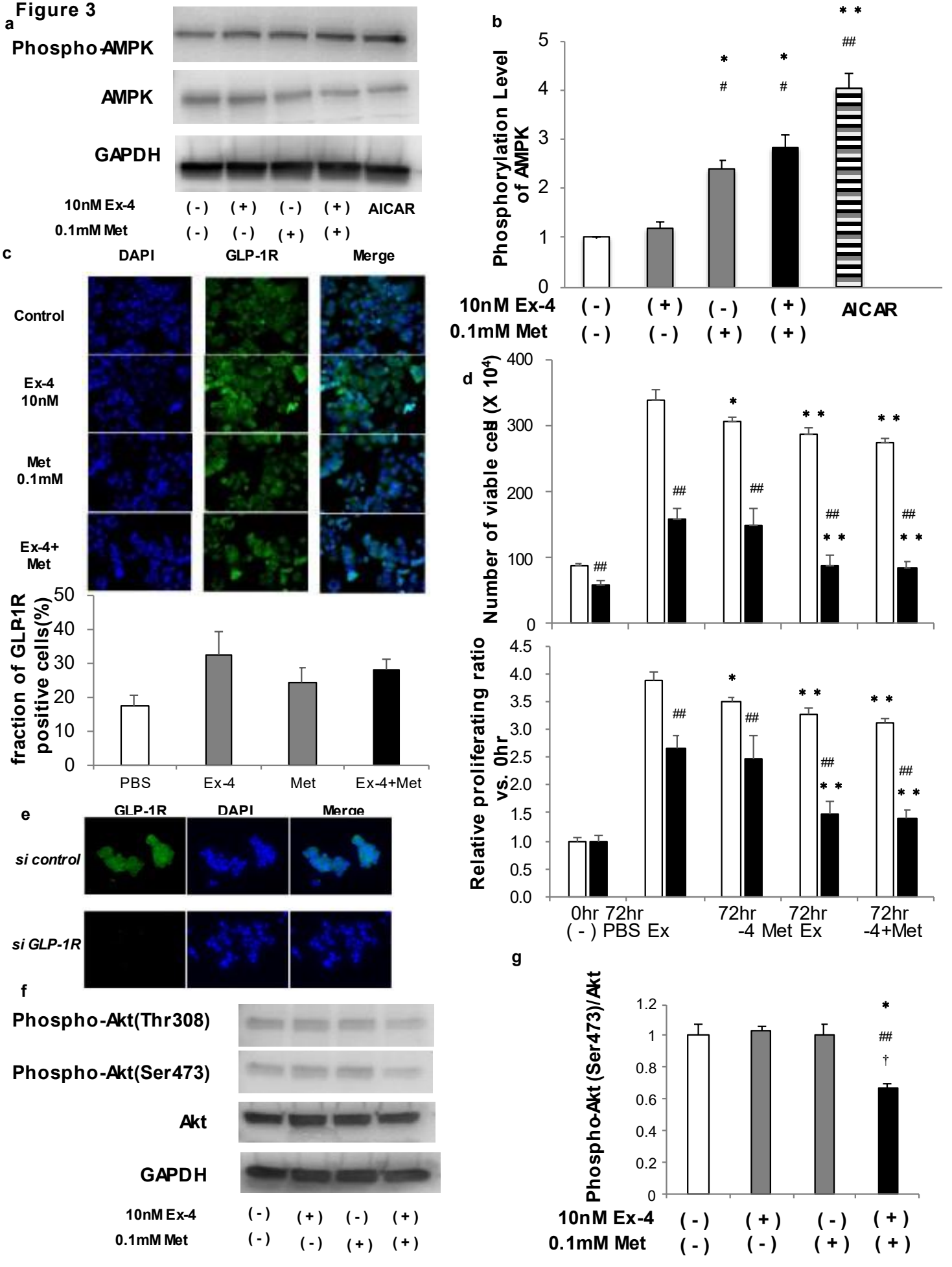
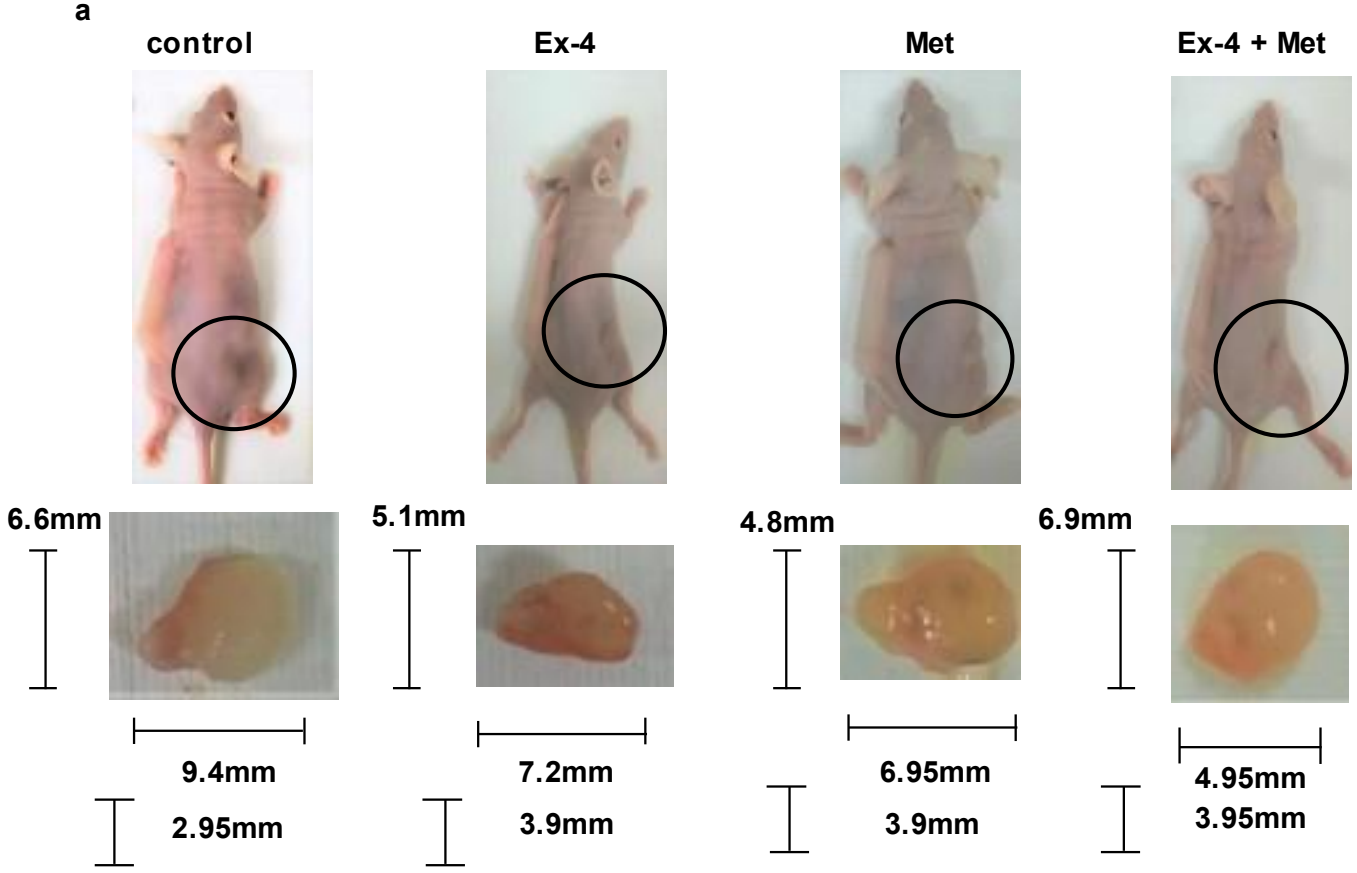
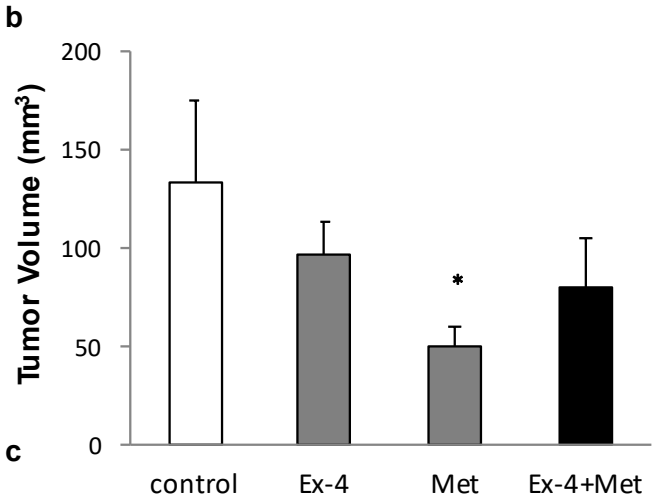
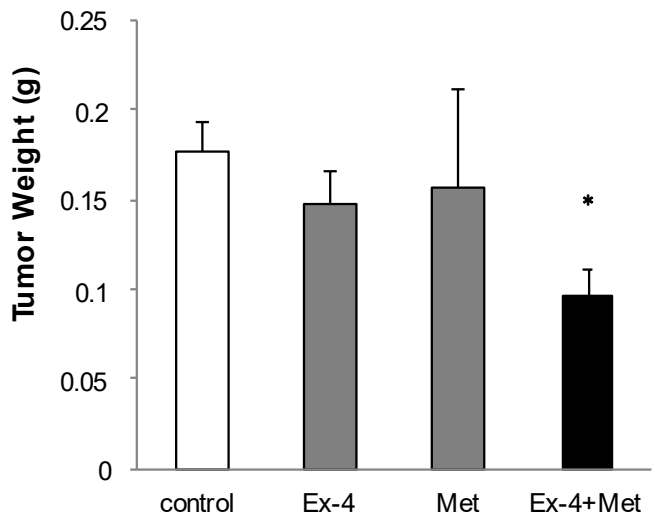
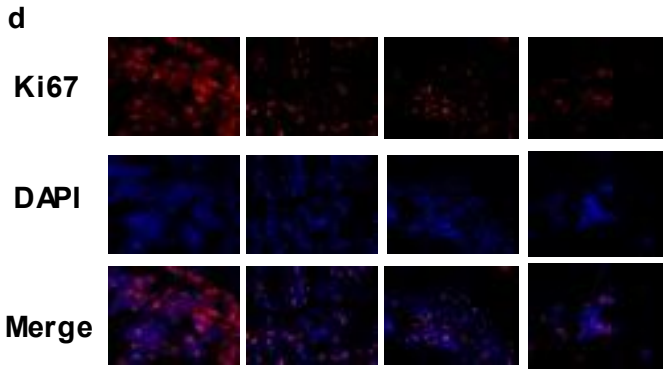

a
Phospho-AMPK
AMPK
GAPDH
10nM Ex-4 (-) (+) (-) (+) AICAR
0.1mM Met (-) (-) (+) (+)
b
Phosphorylation Level of AMPK
0
1
2
3
4
5
10nM Ex-4 (-) (+) (-) (+) AICAR
0.1mM Met (-) (-) (+) (+)
c
DAPI
GLP-1R
Merge
Control
Ex-4 10nM
Met 0.1mM
Ex-4+ Met
fraction of GLP-1R positive cells(%)
0
10
20
30
40
50
PBS
Ex-4
Met
Ex-4+Met
d
Number of viable cell (X 10^4)
0
100
200
300
400
Relative proliferating ratio vs. 0hr
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
0hr 72hr (-) PBS
72hr Ex-4
72hr Met
72hr Ex+Met
e
GLP-1R
DAPI
Merge
si control
si GLP-1R
f
Phospho-Akt(Thr308)
Phospho-Akt(Ser473)
Akt
GAPDH
10nM Ex-4 (-) (+) (-) (+)
0.1mM Met (-) (-) (+) (+)
g
Phospho-Akt (Ser473)/Akt
0
0.2
0.4
0.6
0.8
1
1.2
10nM Ex-4 (-) (+) (-) (+)
0.1mM Met (-) (-) (+) (+)

Figure 4


a
control
Ex-4
Met
Ex-4 + Met
6.6mm
5.1mm
4.8mm
6.9mm
9.4mm
2.95mm
7.2mm
3.9mm
6.95mm
3.9mm
4.95mm
3.95mm


b
Tumor Volume (mm3)
200
150
100
50
0
*
control
Ex-4
Met
Ex-4+Met


c
Tumor Weight (g)
0.25
0.2
0.15
0.1
0.05
0
*
control
Ex-4
Met
Ex-4+Met


d
Ki67
DAPI
Merge

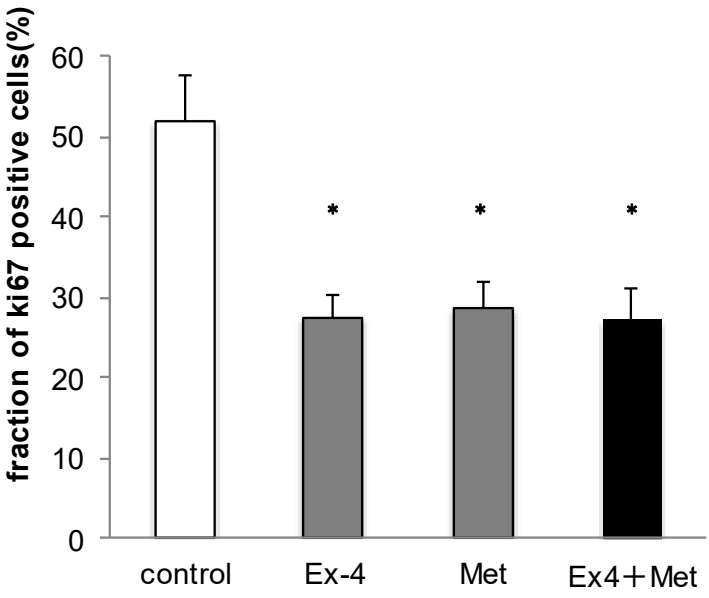
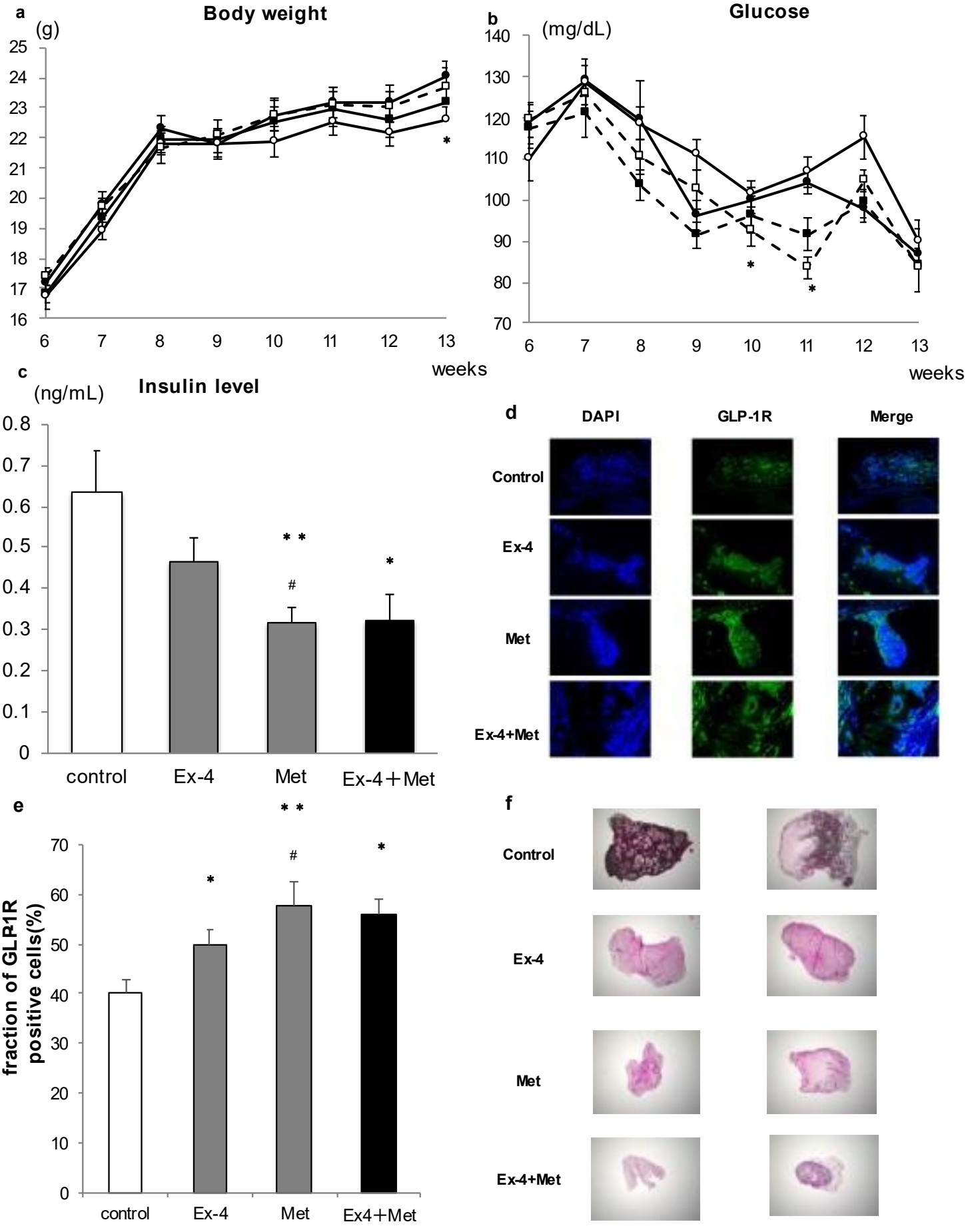

fraction of ki67 positive cells(%)
60
50
40
30
20
10
0
*
*
*
control
Ex-4
Met
Ex4+Met

Figure 5


a
Body weight
(g)
25
24
23
22
21
20
19
18
17
16
6
7
8
9
10
11
12
13
weeks
*
b
Glucose
(mg/dL)
140
130
120
110
100
90
80
70
6
7
8
9
10
11
12
13
weeks
*
*
c
Insulin level
(ng/mL)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
control
Ex-4
Met
Ex-4+Met
* *
#
*
d
DAPI
GLP-1R
Merge
Control
Ex-4
Met
Ex-4+Met
e
fraction of GLR1R positive cells(%)
70
60
50
40
30
20
10
0
control
Ex-4
Met
Ex4+Met
* *
#
*
*
f
Control
Ex-4
Met
Ex-4+Met