

Novel REIC/Dkk-3-encoding adenoviral vector as a promising therapeutic agent for pancreatic cancer

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Momotaro-Gene, Inc. holds the patents for the REIC/DKK3 agent and develops the agent as a cancer therapeutic. M.W., M.S., Y.N., and H.K. own stocks in Momotaro-Gene, Inc.

## **Abstract**

### *Objectives*

Reduced expression in immortalized cells (REIC)/dickkopf 3 (Dkk-3), a tumor suppressor gene, is downregulated in various cancers. We previously reported the tumor-inhibitory effects of the REIC/Dkk-3 gene, delivered by a conventional adenoviral vector (Ad-CAG-REIC,) in pancreatic cancer. Here, we developed an Ad-REIC vector with a novel gene expression system, termed the super gene expression (SGE) system, and assessed its therapeutic effects relative to those of Ad-CAG-REIC in pancreatic cancer cells.

### *Methods*

Human pancreatic cancer cell lines ASPC1 and MIAPaCa2 were used. REIC/Dkk-3 expression was assessed by western blot analysis. Relative cell viability and apoptotic effects were examined in vitro. The anti-tumor effects of Ad-REIC treatment were assessed in the mouse xenograft model.

### *Results*

Compared with Ad-CAG-REIC, Ad-SGE-REIC elicited a significant increase in REIC protein expression in the cells studied. Relative to Ad-CAG-REIC, Ad-SGE-REIC

reduced cell viability and induced apoptosis in the ASPC1 and MIAPaCa2 cell lines in vitro, and achieved superior tumor growth inhibition in the mouse xenograft model.

### *Conclusions*

Compared with conventional Ad-REIC agents, Ad-SGE-REIC provided enhanced inhibitory effects against tumor growth. Our results indicate that Ad-SGE-REIC is an innovative therapeutic tool for pancreatic cancer.

Key words: gene therapy, gene expression, apoptosis

## **Introduction**

Despite recent advances in surgery and aggressive chemotherapy, the prognosis for patients with pancreatic cancer remains poor.<sup>1-5</sup> Only about 20% of patients are considered candidates for surgical resection at the time of diagnosis.<sup>6,7</sup> The overall 5-year survival rate of pancreatic cancer with gemcitabine (GEM), the standard therapy, is less than 5%.<sup>8</sup>

The reduced expression in immortalized cells (REIC) gene, which is identical to Dickkopf-3 (Dkk-3), is a tumor suppressor gene whose expression is downregulated in various cancers including pancreatic cancer.<sup>9,16</sup> In a previous study, we developed an adenoviral vector expressing the human REIC/Dkk-3 gene (Ad-REIC) using cytomegalovirus early enhancer/chicken  $\beta$ -actin (CAG) promoter (Ad-CAG-REIC), and demonstrated that the overexpression of REIC/Dkk-3 induced apoptosis in various cancer cell lines, as well as in pancreatic cancer cell lines.<sup>10-24</sup> Ad-CAG-REIC induces cancer-selective apoptosis by stimulating the unfolded protein response, which results in endoplasmic reticulum (ER) stress and activation of c-Jun-NH2-kinase (JNK).<sup>12</sup> A phase I/IIa study of Ad-CAG-REIC gene therapy for prostate cancer was initiated at Okayama University Hospital in January 2011.<sup>17</sup> In the clinical study, direct and systemic anti-tumor effects induced by Ad-CAG-REIC were found to be strong enough to be

realized as a novel therapeutic agent for castration-resistant prostate cancer.<sup>18</sup> We recently developed a novel gene expression system, termed the super gene expression (SGE) system, and incorporated this system into the Ad-REIC vector in order to construct the Ad-SGE-REIC vector, for even greater therapeutic efficacy. In the Ad-SGE-REIC vector, three specific enhancers of human telomerase reverse transcriptase (hTERT), simian virus 40 (SV40), and cytomegalovirus (CMV) are placed in tandem after the poly A sequence to induce high expression of the inserted REIC gene.<sup>19</sup>

In the current study, we assessed the therapeutic effects of Ad-SGE-REIC, in comparison with Ad-CAG-REIC, in two pancreatic cancer cell lines.

## **Materials and methods**

### ***Cell lines and cell culture***

The human pancreatic cancer cell lines ASPC1 and MIAPaCa2, which were selected because of their similar growth ratios<sup>16</sup>, were obtained from DS Pharma Biochemical Co., Ltd. (Osaka, Japan). ASPC1 and MIAPaCa2 were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA, USA). The media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA), 1% non-essential amino acid (Sigma), 1% sodium pyruvate (Sigma), 1% penicillin/streptomycin solution (Sigma), and amphotericin B (0.5 µg/mL). Cells were cultured at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> and 95% air.

### ***Construction and production of adenoviral vectors***

For construction of the Ad-CAG-REIC vector under the control of the CAG promoter, a full-length complementary DNA of REIC/Dkk-3 was integrated into a cosmid vector, pAxCawt, and transferred into an adenoviral vector according to the COS-TPC method (Takara Bio, Shiga, Japan).<sup>12,19</sup> In the Ad-SGE-REIC vector, hTERT, SV40, and CMV enhancers were placed in tandem after the poly A sequence (Fig. 1) in order to achieve

high expression of the targeted REIC gene.<sup>19</sup> An adenoviral vector carrying the LacZ gene with a CAG promoter (Ad-LacZ) was used as the control vector. The multiplicity of infection (MOI) was defined as the ratio of adenoviral vector-plaque forming units (pfu) to the number of infection-targeted cells. Plaque-based assays were used to determine the pfu titer (pfu/mL). Briefly, a confluent monolayer of HEK293 cells was infected with the virus at varying dilutions and covered with a semi-solid medium of agar and then the pfu titer was determined.<sup>19</sup>

#### ***Western blot analysis***

Cells were plated onto six-well tissue culture plastic dishes and grown to confluence. The cells were then treated with Ad-CAG-REIC and Ad-SGE-REIC at the indicated MOI and cultured for 24 h. The cells were washed twice with cold phosphate-buffered saline (PBS) and lysed with sample buffer (100 mM Tris-HCl [pH 6.8], 10% glycerol, 4% sodium dodecyl sulfate [SDS], 1% bromophenol blue, and 10%  $\beta$ -mercaptoethanol). The samples were then separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA) for western blotting. The membranes were blocked with

PVDF Blocking Reagent (Toyobo, Osaka, Japan) for 1 h. Then, the membranes were incubated for 1 h at room temperature with the following primary antibodies: human phospho-inositol-requiring enzyme 1 $\alpha$  (IRE1 $\alpha$ ) (S724) (#ab48187 from Abcam, Cambridge, MA, USA), human phospho-stress-activated protein kinase/Jun aminoterminal kinase (SAPK/JNK) (Thr183/Tyr185) (#4668 from Cell Signaling Technology, Danvers, MA), human phospho-c-Jun (Ser73) (#3270 from Cell Signaling Technology), cleaved caspase-3 (Asp175) (#9661 from Cell Signaling Technology), human  $\beta$ -actin (#4967 from Cell Signaling Technology), and mouse monoclonal anti-human REIC/Dkk-3 antibody (produced in our laboratory). After three washes with Tris-buffered saline with Tween-20 (Sigma), the membranes were incubated with secondary antibody for 1 h at room temperature. The chemiluminescence images were obtained using a LAS 4000 imager (GE Healthcare Life Sciences, Pittsburgh, PA, USA). The signal intensity was determined using ImageQuant software (GE Healthcare Life Sciences, Pittsburgh, PA, USA).

### ***Cell viability assay***

The cells ( $2 \times 10^5$  cells) were seeded in flat-bottomed six-well plates and incubated

for 24 h. The cells were treated with Ad-LacZ, Ad-CAG-REIC, or Ad-SGE-REIC at the indicated MOI in 0.5 ml of serum-free medium for 1 h. Then, 1.5 ml of fresh medium was added and cells were incubated for 72 h. Cell viability was assessed using 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, yellow tetrazole (MTT) to determine whether REIC/Dkk-3 expression affects cell growth in the pancreatic cancer cell lines ASPC1 and MIAPaCa2. Next, 200  $\mu$ l of MTT was added to each well, and the cells were incubated for an additional 4 h at 37°C. The purple-blue formazan precipitate was dissolved using DMSO, and then 100  $\mu$ l of the solution was added to flat-bottomed 96-well microplates. Cell viability was evaluated by measuring the absorbance at 570 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

### ***Apoptosis assay***

The cells ( $2 \times 10^5$  cells) were seeded in flat-bottomed six-well plates and incubated for 24 h. The cells were treated with Ad-LacZ, Ad-CAG-REIC or Ad-SGE-REIC at the indicated MOI in 0.5 ml of serum-free medium for 1 h. Then, 1.5 ml of fresh medium was added and the cells were incubated for 72 h. Hoechst 33342 (Wako, Tokyo, Japan) was added to the medium at a concentration of 2  $\mu$ g/mL, and the cells were incubated in

the dark for 10 min.<sup>15</sup> Apoptotic cells were counted under a microscope, and the percentage of apoptotic cells for each sample was determined. One hundred cells were examined per field, and the average percentage of apoptotic cells in five different fields was calculated.

### ***Tumor growth assay in the mouse xenograft model***

ASPC1 cells ( $5.0 \times 10^6$  in 100  $\mu\text{L}$  of PBS) were injected subcutaneously into the left flanks of 8-week-old BALB/c female mice (SLC, Hamamatsu, Japan). The mice were then randomly assigned to treatment groups of five animals each. One week after the injection of ASPC1 cells,  $1.0 \times 10^9$  plaque forming units (pfu) of Ad-LacZ, Ad-CAG-REIC, or Ad-SGE-REIC in 100  $\mu\text{L}$  of PBS were injected intra- and peri-tumorally. The day of adenovirus vector injection was designated day 0. Adenovirus vectors were injected at days 7, 14, 21, and 28 at a dose of  $1.0 \times 10^9$  pfu /100  $\mu\text{L}$  PBS/ injection. Tumor size was measured every week for 5 weeks after injection, and mice were sacrificed on day 35. Tumor volume was calculated using the following empirical formula:  $V = 1/2 \times [(the\ shortest\ diameter)^2 \times (the\ longest\ diameter)]$ . Mice were treated and cared for in strict accordance with the guidelines of

the Institutional Animal Care and Use Committee of Okayama University Faculty of Medicine.

### *Statistical analysis*

Results are presented as means  $\pm$  standard error (SE). Each in vitro experiment was performed independently at least three times. Differences were evaluated using the **two-tailed** Student's t-test. A p value of less than 0.05 was considered statistically significant.

## **Results**

### ***The SGE system efficiently enhanced REIC/Dkk-3 gene expression***

We developed an adenoviral vector, harboring the SGE cassette, for expression of the REIC/Dkk-3 gene (Fig. 1).<sup>19</sup> In order to demonstrate the enhanced gene expression induced by the SGE system, we compared REIC/Dkk-3 protein expression levels in the transduced human pancreatic cancer cell lines (ASPC1 and MIAPaCa2) using western blot. It was found that Ad-SGE-REIC-transduced cells exhibited a significant increase (2.0-fold and 1.8-fold in ASPC1 and MIAPaCa2 cell lines, respectively) in REIC protein expression (Fig. 2).

### ***Ad-SGE-REIC induced apoptosis more efficiently than Ad-CAG-REIC***

Ad-CAG-REIC treatment reduced cell viability by  $62.0 \pm 4.5\%$  and  $39.6 \pm 1.6\%$  in the ASPC1 and MIAPaCa2 cell lines, respectively, in comparison with Ad-LacZ treatment (Fig. 3A). Ad-SGE-REIC treatment markedly reduced cell viability by  $83.1 \pm 2.2\%$  and  $67.2 \pm 4.2\%$  in ASPC1 and MIAPaCa2 cells, respectively (Fig. 3A). We have previously found that REIC/Dkk-3 reduces cell viability by inducing apoptosis.<sup>12-16</sup> Therefore, apoptosis assay was performed using Hoechst 33342 dye in order to

determine the increment of apoptotic cells following treatment with Ad-CAG-REIC (ASPC1;  $40.4 \pm 1.7\%$ , MIAPaCa2;  $38.8 \pm 3.5\%$ ) and Ad-SGE-REIC (ASPC1;  $73.9 \pm 1.5\%$ , MIAPaCa2;  $70.0 \pm 0.6\%$ ) (Fig. 3B).

#### ***Ad-SGE-REIC treatment induced apoptosis via the JNK signaling pathway***

REIC/Dkk-3 overexpression has been reported to induce apoptosis by increasing ER stress in pancreatic cancer cells, as well as in those of other cancer types.<sup>12-16</sup> Therefore, we examined ER stress-related activation of the JNK signaling pathway. Western blot analysis demonstrated that phosphor-IRE $\alpha$ , phosphor-JNK, and phosphor-c-Jun expression was activated by Ad-CAG-REIC treatment (Fig. 4). The expression of these signaling molecules was more intensely activated in cells treated with Ad-SGE-REIC (Fig. 4).

#### ***Intratumoral Ad-SGE-REIC administration strongly inhibited tumor growth in the mouse xenograft model.***

In order to evaluate the therapeutic utility of Ad-SGE-REIC, we administered Ad-SGE-REIC intratumorally in the mouse xenograft model using ASPC1 cells. We

found that Ad-CAG-REIC treatment suppressed tumor growth by  $41.4 \pm 9.8\%$  (Fig. 5); however, this reduction was not statistically significant. Treatment with Ad-SGE-REIC resulted in stronger inhibition of tumor growth (by  $60.7 \pm 9.9\%$ ,  $p < 0.05$ ) in comparison with Ad-LacZ treatment. (Fig. 5)

## **Discussion**

Surgical resection is considered the only curative therapy for patients with pancreatic cancer; however, only 20% of patients are considered suitable candidates for surgical resection at the time of diagnosis.<sup>6,7</sup> Patients with advanced pancreatic cancer exhibit a median survival time of approximately 6 months, and a 5-year overall survival rate of less than 5%.<sup>4,6,7,25,26</sup> The prognosis of patients with pancreatic cancer is poor, and new therapies are required to treat such patients.

REIC/Dkk3 is a tumor suppressor gene whose expression is downregulated in various cancers.<sup>9</sup> We developed an adenoviral vector expressing the human REIC/Dkk-3 gene under the control of the CAG promoter.<sup>19-22</sup> In a previous preclinical study, we found that the overexpression of REIC/Dkk-3, encoded by the Ad-CAG-REIC vector, induced apoptosis in various cancer cell lines,<sup>10-15</sup> thereby demonstrating the potential utility of Ad-CAG-REIC gene therapy for the treatment of pancreatic cancer.<sup>16</sup> However, a strong level of gene expression is required to achieve greater therapeutic efficacy. Therefore, in the present study, we developed an Ad-REIC vector incorporating the SGE system by placing the triple translational enhancer sequences of hTERT, SV40, and CMV downstream of the BGH poly A sequence (Fig. 1).<sup>19</sup> Additionally, we assessed the therapeutic effects of Ad-SGE-REIC, in comparison with those of Ad-CAG-REIC,

against pancreatic cancer. First, we assessed the efficiency of REIC protein expression in pancreatic cancer cells. In accordance with the findings of previous studies in other cancers,<sup>19</sup> Ad-SGE-REIC induced REIC protein expression more efficiently (Fig. 2). Ad-CAG-REIC inhibits cell proliferation by inducing apoptosis in pancreatic cancer cells.<sup>16</sup> Therefore, we performed an MTT assay and an apoptosis assay in order to evaluate the induction of apoptosis in Ad-SGE-REIC-treated cells. As expected, treatment with Ad-SGE-REIC reduced cell proliferation by inducing apoptosis with greater efficiency than that achieved by Ad-CAG-REIC treatment (Fig. 3).

The overexpression of REIC/Dkk-3 encoded by the Ad-CAG-REIC vector results in ER-stress-mediated JNK activation, inducing apoptosis and thereby inhibiting tumor growth, in Ad-CAG-REIC-treated pancreatic cancer cells.<sup>12,16,24</sup> Ad-SGE-REIC utilizes the same mechanism during activation of the JNK pathway and induction of ER stress, but with greater efficiency (Fig. 4).

In order to confirm the therapeutic effect of Ad-SGE-REIC, we constructed a mouse xenograft model with ASPC1 cells. Treatment with Ad-SGE-REIC elicited stronger anti-tumor effects than with Ad-CAG-REIC (Fig. 5). These findings demonstrate that Ad-SGE-REIC-based therapy represents a promising therapeutic strategy for the treatment of pancreatic cancer. Several potential strategies involving gene therapy have

been reported for the treatment of pancreatic cancer<sup>27-33</sup>; however, these have achieved only limited success. In a previous study, we demonstrated the therapeutic effects of Ad-CAG-REIC in combination with GEM.<sup>16</sup> Treatment with Ad-CAG-REIC was found to inhibit tumor growth by 25.0% as compared with Ad-LacZ treatment at 28 days, whereas Ad-CAG-REIC inhibited tumor growth by 55.0% in combination with GEM. Ad-SGE-REIC treatment without GEM demonstrated an equivalent tumor-suppression effect ( $50.0 \pm 13.0\%$  at 28 days). These findings suggest that single administration of Ad-SGE-REIC shows potential utility as a therapeutic agent for pancreatic cancer.

REIC-induced anticancer immune activation has been demonstrated in previous studies.<sup>17,24,34</sup> We did not assess anti-cancer immunity induced by Ad-SGE-REIC, as the xenograft mouse model used in the present study lacks an immunocompetent innate immune system. Further study is necessary to assess the anti-cancer immunity induced by Ad-SGE-REIC using an immune-competent pancreatic cancer mouse model.

Endoscopic ultrasound delivery (EUS) of an anti-tumor agent represents an attractive method for delivery of therapeutic agents in the treatment of pancreatic cancer. Accordingly, several clinical trials to test the efficacy of EUS-guided anti-tumor agents e.g. ONYX-015 and HF10, are currently ongoing.<sup>27,31,35</sup> In addition, percutaneous injection therapy, which has already been developed as a safe drug delivery method for

the treatment of liver tumors,<sup>36</sup> represents an alternative delivery method for therapeutic agents in patients who have pancreatic cancer and have also developed liver metastasis. In the present study, we found that the Ad-SGE-REIC vector significantly enhances REIC/Dkk-3 protein expression and exerts a much stronger cell-killing effect in pancreatic cancer cells in comparison with the conventional Ad-CAG-REIC agents. Ad-SGE-REIC treatment additionally achieved significant therapeutic effects in xenograft mice with pancreatic cancer cells, indicating that Ad-SGE-REIC-based gene therapy represents an innovative therapeutic strategy for the treatment of patients with pancreatic cancer.

**Conflicts of Interest and Source of Funding:**

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Momotaro-Gene, Inc. holds the patents for the REIC/DKK3 agent and develops the agent as a cancer therapeutic. M.W., M.S., Y.N., and H.K. own stocks in

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## Figure Legends

Figure 1.

Construction of the Ad-SGE-REIC vector

Schematic representation of the conventional gene expression system and the SGE system. In the conventional gene expression system, cytomegalovirus (CMV) or CMV early enhancer/chicken  $\beta$ -actin (CAG) promoter has been used to increase the gene expression system. In the SGE system, three specific enhancers of human telomerase reverse transcriptase (hTERT), simian virus 40 (SV40), and CMV are placed in tandem after the poly A sequence.<sup>19</sup>

Figure 2.

Western blot analysis of REIC/Dkk-3 protein expression.

A western blot analysis was performed in order to compare REIC/Dkk-3 protein expression levels after transfection of ASPC1 and MIAPaCa2 human cancer cell lines with Ad-CAG-REIC and Ad-SGE-REIC at 30 MOI. Western blot for  $\beta$ -actin levels was performed to verify equal loading of cellular proteins. Representative blots of more than three independent experiments are shown. The graph below the blot shows the results of

signal intensity analysis from three independent western blotting experiments. The results are presented as the mean  $\pm$  standard error (SE) (n = 3). \*P < 0.05.

Figure 3.

Comparison of the effects of treatment with Ad-SGE-REIC and Ad-CAG-REIC.

(a) The cell proliferation rate was examined by MTT assay following the indicated treatment (no treatment, Ad-LacZ treatment, Ad-CAG-REIC treatment, or Ad-SGE-REIC treatment at 100 MOI for 72 h) in ASPC1 and MIAPaCa2 cells.

(b) The rate of apoptosis was examined by Hoechst 33342 staining following the indicated treatment (no treatment, Ad-LacZ treatment, Ad-CAG-REIC treatment, or Ad-SGE-REIC treatment at 100 MOI for 72 h) in ASPC1 and MIAPaCa2 cells.

The results are presented as the means  $\pm$  standard error (SE) (n = 4). \*P < 0.05.

Figure 4.

Western blot analysis to evaluate activation of signaling molecules of the JNK signaling pathway.

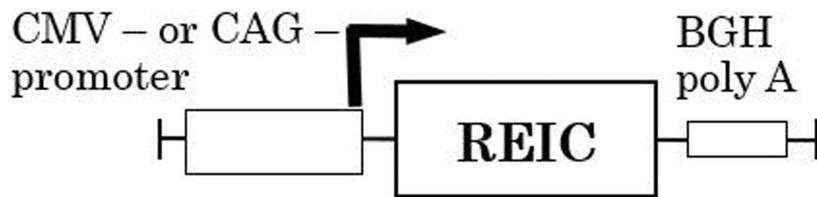
A Western blot analysis was performed to evaluate activation of signaling molecules of the JNK signaling pathway by Ad-SGE-REIC in comparison with Ad-CAG-REIC. Cells were transfected with Ad-LacZ, Ad-CAG-REIC, and Ad-SGE-REIC at 30 MOI. Cell lysates were collected 48 h after transfection. The protein expression of phospho-IRE1 $\alpha$ , phospho-JNK, phospho-c-Jun, and  $\beta$ -actin was assessed by western blot analysis using specific antibodies. Western blot analysis for  $\beta$ -actin was performed to verify equal loading. An analysis of the signal intensities of three independent western blot experiments is shown in the graph below the blot. The results are presented as the means  $\pm$  standard error (SE) (n = 3). \*P < 0.05.

Figure 5.

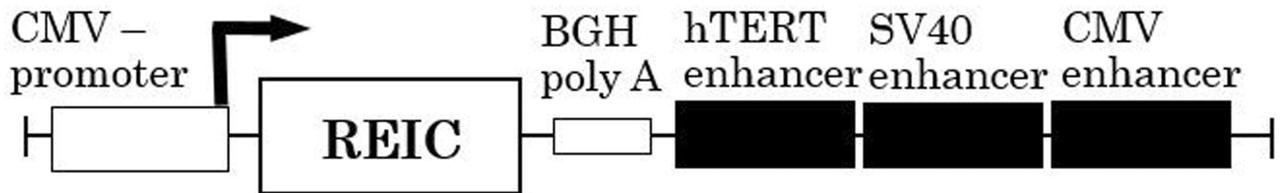
Effect of Ad-SGE-REIC treatment in the mouse pancreatic cancer xenograft model.

The inoculated mice were divided into three treatment groups (Ad-LacZ, Ad-CAG-REIC, and Ad-SGE-REIC). The tumor volume was calculated as described in the Materials and Methods section. The results are presented as the means  $\pm$  standard error (SE) (n = 5). \*P < 0.05.

## Conventional gene expression system



## Super gene expression (SGE) system



ASPC1

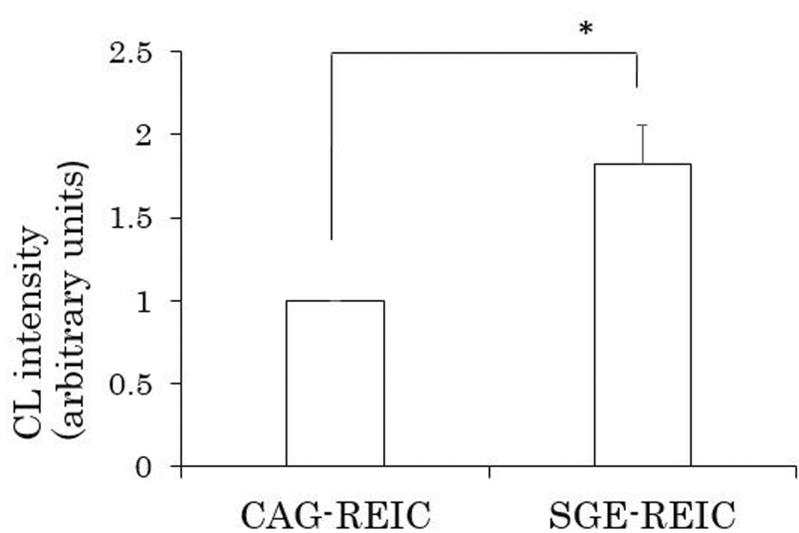
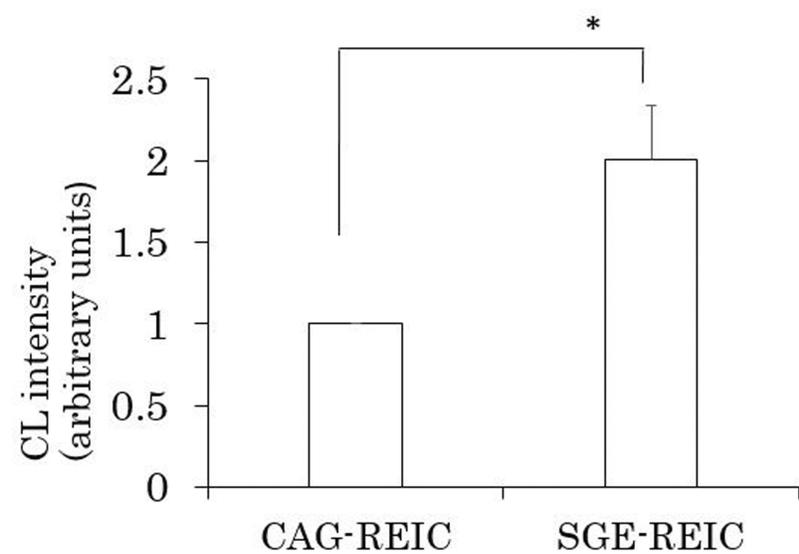
MIAPaCa2

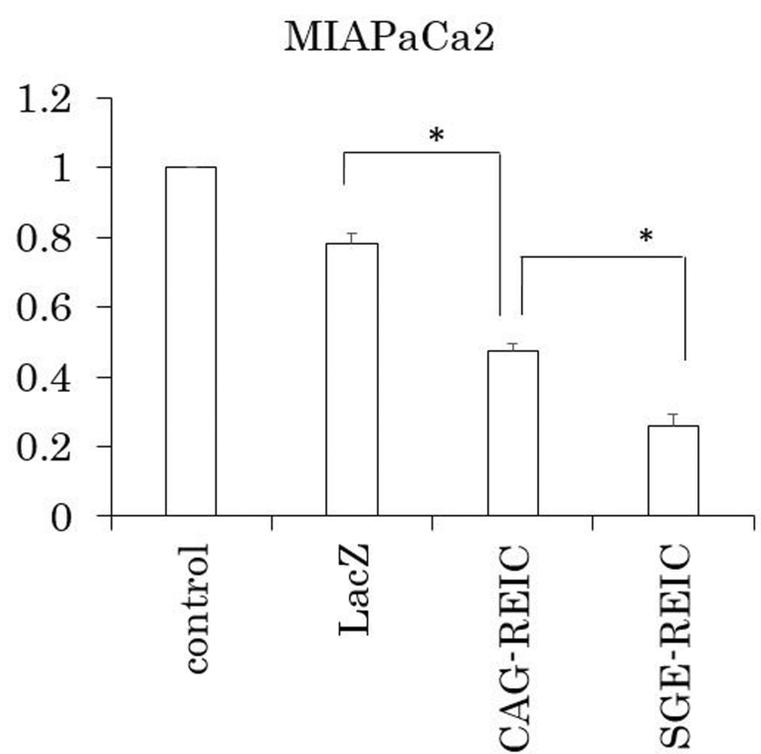
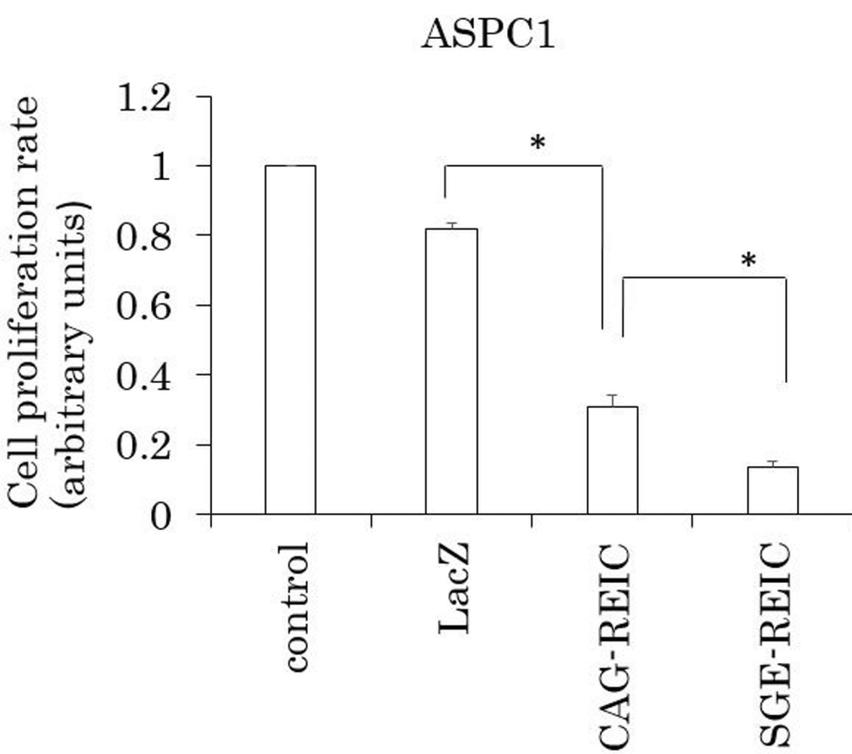
LacZ CAG-REIC SGE-REIC

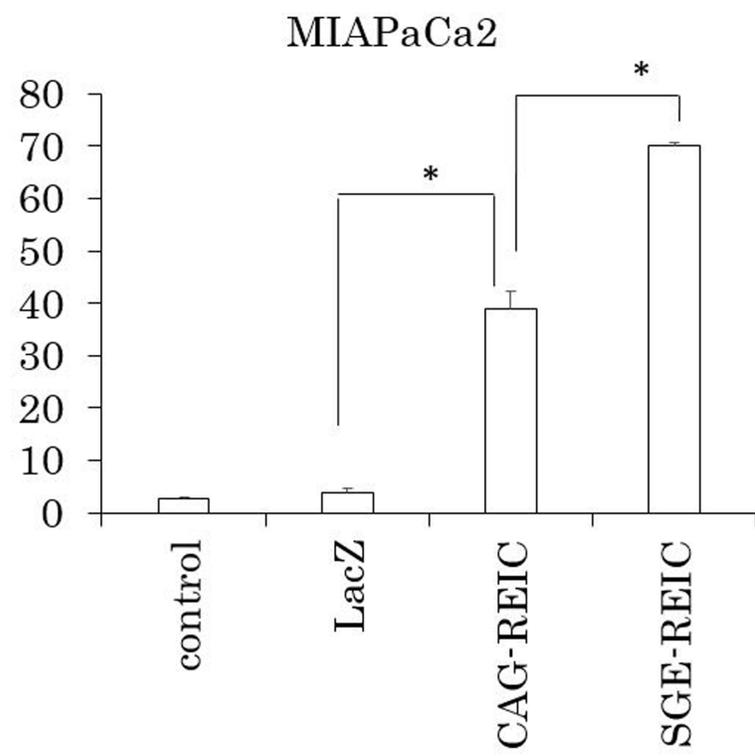
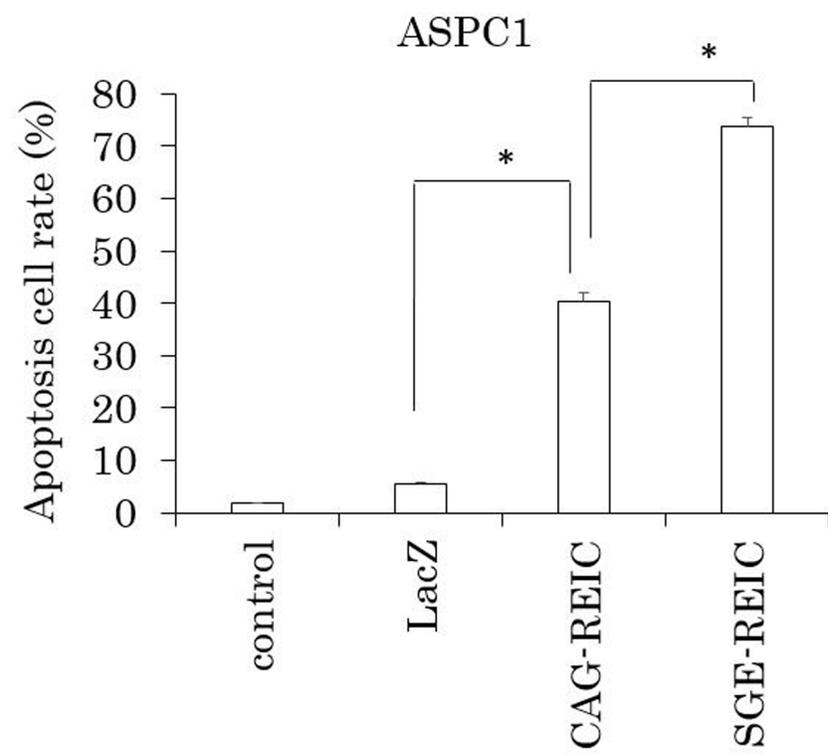
LacZ CAG-REIC SGE-REIC

REIC

$\beta$ -actin







## ASPC1

## MIAPaCa2

