Carrier cell-mediated cell lysis of squamous cell carcinoma by squamous cell carcinoma antigen 1 promoter-driven oncolytic adenovirus

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Abstract

The squamous cell carcinoma antigen (SCCA) serves as a serological marker for squamous cell carcinomas. Molecular cloning of the *SCCA* genomic region has revealed the presence of two tandemly arrayed genes, *SCCA1* and *SCCA2*. We examined the promoter activity of the 5'-flanking proximal region of the *SCCA1* gene. Deletion analysis of *SCCA1* promoter identified a 175-bp core promoter region and an enhancer region at -525 to -475 bp upstream of the transcription start site. The transcriptional activity of the *SCCA1* promoter was up-regulated in squamous cell carcinoma cells, compared with normal keratinocyte, normal non-keratinocyte and adenocarcinoma cells. Five tandem repeats of enhancer increased *SCCA1* promoter activity by 4-fold. Oncolytic adenovirus driven by the *SCCA1* promoter with 5 tandem repeats of enhancer specifically killed squamous cell carcinoma cells *in vitro* and *in vivo*. A549 carrier cells infected with the oncolytic adenovirus induced complete regression of tumor by overcoming immunogenicity and adenovirus-*mGM-CSF* augmented the antitumor effect of carrier cells. These findings suggest that *SCCA1* promoter is a potential target of gene therapy for squamous cell carcinoma.

Introduction

Squamous cell carcinoma antigen (SCCA) is a circulating tumor marker for squamous cell carcinoma, especially that of cervix, head and neck, lung, and esophagus.[1] Elevated circulating levels of SCCA are not detected in patients with adenocarcinomas of the uterus, ovary, or breast.[2] Several studies have shown that increased serum SCCA levels are correlated with extent of disease in patients with squamous cell carcinoma.[2-4] Higher SCCA levels are also indicative of deep tumor infiltration and lymph node involvement.[5, 6] Moreover, measurement of post-treatment SCCA levels is useful for monitoring the response to therapy and for predicting tumor recurrence and metastasis. SCCA protein has been isolated from a metastatic, cervical squamous cell carcinoma.[2] Molecular cloning of the *SCCA* genomic region revealed the presence of two tandemly arrayed genes, *SCCA1* and *SCCA2*. Although SCCA1 and SCCA2 are nearly identical members of the serpin superfamily, the significant differences in their reactive site loops suggest that SCCA1 is a papain-like cysteine proteinase inhibitor, while SCCA2 is a chymotrypsin-like serine proteinase inhibitor.[7]

Previous studies have reported the cloning and characterization of the promoter region of *SCCA1*[8] and *SCCA2*.[9] *SCCA1* gene expression and promoter activity is up-regulated in squamous cell carcinoma cells, compared with keratinocyte and adenocarcinoma cells.[8, 10] *SCCA2* gene expression and promoter activity are also increased in squamous cell carcinoma cells compared with normal and adenocarcinoma cells, though the *SCCA1* gene expression and promoter activity are higher than the *SCCA2* gene expression in almost all squamous carcinoma cells and tissues.[8, 10] These findings suggest that *SCCA1* promoter may be a potential target of

gene therapy for squamous cell carcinoma. Although the *SCCA2* promoter has been introduced into E1-deleted adenovirus to transduce an apoptotic gene in lung cancer[11], use of *SCCA1* promoter-driven adenovirus to treat squamous cell carcinoma has not yet been reported. We found that up to 5 consecutive tandem-repeat enhancer elements significantly increased *SCCA1* promoter activity. Oncolytic adenovirus AdE3-*SCCA1* was constructed by replacement of adenovirus *E1A* promoter with this tandem-repeat enhancer and promoter complex and specifically killed squamous cell carcinoma cells.

Results

Expression of SCCA protein

To determine the levels of expression of SCCA protein in human cervical squamous cell carcinoma cells, ELISA was performed in the medium of each cell line. SKGIIIa and HT-III cells secreted the highest concentrations of SCCA proteins into medium (**Fig. 1**). The mean SCCA protein level secreted by cervical squamous cell carcinoma cells was 15 times that by keratinocyte cells, 404 times that by normal non-keratinocyte cells, and 329 times that by adenocarcinoma cells. The mean SCCA protein level secreted by keratinocyte cells was 28 times that by normal non-keratinocyte cells, and 23 times that by adenocarcinoma cells. The mean SCCA protein level secreted by normal non-keratinocyte cells was not significantly different from that by adenocarcinoma cells.

mRNA levels of *SCCA1* and *SCCA2* in cervical squamous cell carcinoma cells

To examine the mRNA levels of *SCCA1* and *SCCA2* in cervical squamous cell carcinoma cells, one-step real time RT-PCR was performed using MGB probe. The mRNA level of *SCCA1* was one or two order of magnitude higher than that of *SCCA2* in all cell types examined (**Fig. 2**). The mRNA level of *SCCA1* in cervical squamous cell carcinoma cells was 8.5, 280, and 195 times those in normal keratinocyte, normal non-keratinocyte, and adenocarcinoma cells, respectively. The mRNA level of *SCCA1* in normal keratinocyte cells was 33 and 23 times those in normal non-keratinocyte and adenocarcinoma cells, respectively. The mRNA level of *SCCA1*

in normal non-keratinocyte cells was not significantly different from that in adenocarcinoma cells.

Transcriptional activities of *SCCA1* and *SCCA2* in squamous cell carcinoma cells

To examine the transcriptional activities of *SCCA1* in squamous cell carcinoma cells, transient expression assays were performed. Luciferase reporter plasmids containing varying lengths of the 5'-flanking regions of the gene were constructed (SCCA1-750, SCCA1-550, SCCA1-525, SCCA1-500, SCCA1-475, SCCA1-450, SCCA1-375, SCCA1-250, SCCA1-175, SCCA1-125, and SCCA1-75) as shown in **Fig. 3a** and transfected into HT-III cells, and cell lysates were tested in luciferase assays. **Fig. 3b** demonstrates the transcriptional activities in HT-III cells. Deletion analysis from the -750-bp to -525-bp region upstream from the gene revealed gradual increase in transcriptional activity with decrease in length, suggesting the presence of a sequence inhibiting transcriptional activities of the gene between -750-bp and -525-bp. A region from -525-bp to -475-bp of the *SCCA1* promoter demonstrated significant transcriptional activity. Deletion analysis from the -450-bp to -175-bp region upstream from the gene revealed gradual increase in transcriptional activity with decrease in length of promoter, suggesting the presence of a sequence inhibiting transcriptional activities of the gene between -450-bp and -250-bp. A -175-bp region of *SCCA1* promoter demonstrated significant transcriptional activity. This proximal negative region from -525-bp to -475-bp and positive -175-bp region have not reported previously.[8]

To determine the enhancer region, the intron 1 region or proximal promoter region from -525-bp to -475-bp was inserted into upstream of the -175-bp region of *SCCA1* luciferase promoter plasmid. Luciferase reporter plasmids of SCCA1-175 containing intron 1 in sense or antisense orientation or the region from -525-bp to -475-bp were constructed (INT-SCCA1-175 for sense orientation of intron 1, INT-AS-SCCA1-175 for antisense orientation of intron 1, 525-SCCA1-175 for sense orientation of -525-bp to -475-bp , 525-AS-SCCA1-175 for antisense orientation of -525-bp to -475-bp, 525x2-SCCA1-175, 525x3-SCCA1-175, 525x4-SCCA1-175, 525x5-SCCA1-175, 525x10-SCCA1-175, and 525x20-SCCA1-175 for tandem repeats of -525-bp to -475-bp) as shown in **Fig. 4a** and transfected into HT-III cells, and cell lysates were tested in luciferase assays. **Fig. 4b** demonstrates the transcriptional activities in HT-III cells. Intron 1 did not enhance SCCA1-175 promoter activity, consistent with a previous report.[13] However, the sense and antisense orientations of the -525-bp to -475-bp region significantly enhanced SCCA1-175 promoter activity, indicating that this region functioned as an enhancer for the SCCA1-175 promoter. Five tandem repeats of the -525-bp to -475-bp region increased SCCA1-175 promoter activity up to four-fold, while 10 or 20 tandem repeats of this enhancer region did not further increase SCCA1-175 promoter activity compared with 5 tandem repeats.

To compare the tissue specificity of the transcriptional activity of enhancer-promoter complex of *SCCA1* promoter, various numbers of tandem repeats of enhancer and SCCA1-175 promoters were transfected into HT-III squamous cell carcinoma cells and H1299 adenocarcinoma cells. The promoter activity of SCCA1-175 in HT-III cells was 20 times that in H1299 cells and the promoter activity of 5 tandem repeats of the enhancer and SCCA1-175 in

HT-III cells was 37 times that in H1299 cells (**Fig. 4c**). Thus, 5 tandem repeats of enhancer and promoter complex exhibited significant tissue specificity in squamous cell carcinoma cells compared with adenocarcinoma cells.

To compare the tissue specificity of transcriptional activity of the enhancer-promoter complex of *SCCA1* promoter, SCCA1-175 with 5 tandem repeats of enhancer and SCCA1-175 promoter were transfected into squamous cell carcinoma cells, normal keratinocyte, normal non-keratinocyte cells, and adenocarcinoma cells. The promoter activity of SCCA1-175 in squamous cell carcinoma cells was 2.4 times that in keratinocyte cells, 7.6 times that in normal non-keratinocyte cells, and 13 times that in adenocarcinoma cells (**Fig. 4d**). The promoter activity of SCCA1-175 in normal keratinocytes was 3 times that in non-keratinocyte cells, and 5 times that in adenocarcinoma cells. The promoter activity of 5 tandem repeats of enhancer and SCCA1-175 in squamous cell carcinoma cells was 5 times that in keratinocytes, 16 times that in non-keratinocyte cells, and 30 times that in adenocarcinoma cells. The promoter activity of 5 tandem repeats of enhancer and SCCA1-175 in keratinocytes was 3 times that in non-keratinocyte cells, and 6 times that in adenocarcinoma cells. The promoter activities of SCCA1-175 and SCCA1-175 with tandem repeats of enhancer did not differ significantly between normal non-keratinocyte cells and adenocarcinoma cells. Thus, 5 tandem repeats of enhancer significantly increased SCCA1-175 promoter activity in squamous cell carcinoma cells compared with normal keratinocytes, normal non-keratinocyte cells, and adenocarcinoma cells.

Transcriptionally targeted AdE3-*SCCA1* has a potent antiproliferative effect in squamous cell carcinoma cells but not in normal non-keratinocyte cells or adenocarcinoma cells

To estimate the potential of *SCCA1* promoter for use in gene therapy for squamous cell carcinoma, *SCCA1* promoter-driven oncolytic adenovirus was constructed and transfected into each cell line. **Fig. 5a** shows the construct of oncolytic adenovirus AdE3-*SCCA1* in which the 404-551bp region of *E1A* promoter was substituted by 5 tandem repeats of enhancer and SCCA1-175 promoter complex. **Fig. 5b** shows the growth-inhibitory effects (IC50) of oncolytic adenovirus AdE3-*SCCA1* in various types of cell lines. The IC50 of wild-type adenovirus AdE3 did not differ significantly among squamous cell carcinoma cells, normal keratinocytes, normal non-keratinocyte cells nor adenocarcinoma cells. AdE3-*SCCA1* killed neither normal non-keratinocyte cells nor adenocarcinoma cells. AdE3-*SCCA1* significantly suppressed the growth of normal keratinocytes compared with normal non-keratinocyte cells and adenocarcinoma cells. Furthermore, this growth-inhibitory effect of AdE3-*SCCA1* was significantly enhanced in squamous cell carcinoma cells, and was one or two orders of magnitude higher than in normal keratinocytes.

Oncolytic adenovirus AdE3-*SCCA1* suppresses subcutaneous tumor growth of squamous cell carcinoma in nude and syngeneic mice

To evaluate the antitumor effect of AdE3-*SCCA1*, xenograftic subcutaneous tumors were established in the flanks of nude mice using cervical cancer HT-III and adenocarcinoma H1299 cells. By 30 days, we observed significant reduction in tumor size in the AdE3-*SCCA1*- and AdE3-treated groups compared with the medium alone- and Ad5CMV-*LacZ*-treated groups in

the HT-III tumor models. AdE3-*SCCA1* and AdE3 significantly reduced tumor sizes by 95-98% compared with medium alone and Ad5CMV-*LacZ* ($P < 0.001$, unpaired *t*-test) (**Fig. 6a**). In contrast, AdE3-*SCCA1* did not significantly reduce the sizes of H1299 tumors compared with Ad5CMV-*LacZ* (**Fig. 6b**). However, AdE3 significantly reduced the size of H1299 tumors by 96% compared with Ad5CMV-*LacZ* ($P < 0.001$, unpaired *t*-test).

To evaluate the antitumor effects of AdE3-*SCCA1* after immunization, C3H mice were immunized with Ad5CMV-*LacZ* and subcutaneous tumors 5-8 mm in diameter were established in the left thigh of C3H mice using mouse squamous carcinoma SCC7 cells (**Fig. 6c**). Survival of control mice was not significantly different from that of the mice treated with AdE3-*SCCA1* alone. Survival of mice treated with A549 carrier cells infected with AdE3-*SCCA1* was significantly longer than those of the mice treated with medium control or AdE3-*SCCA1*. Furthermore, simultaneous infection with AxCA*mGM-CSF* augmented the antitumor effect of A549 carrier cells infected with AdE3-*SCCA1* ($P < 0.05$). Mice that exhibited complete tumor regression were resistant to subsequent inoculation of SCC7 cells.

Discussion

In the present study, the highest *SCCA1* promoter activities were found in the 175-bp region just upstream of the *SCCA1* gene. It has been reported that a 0.5-kb fragment upstream of the *SCCA1* gene exhibited significant promoter activity.[8] This is consistent with the enhancer region and promoter activity identified in the present study. The negative element in the region 0.3- to 0.4-kb upstream of the *SCCA1* gene was reported previously,[12] and corresponds to the negative 250- to 450-bp region in the present study. The positive 175-bp *SCCA1* promoter element had not yet been previously reported because the promoter activity of the 0- to 200-bp fragment of the *SCCA1* gene remained to be determined. The present study is thus the first to report promoter analysis of the proximal region of *SCCA1* promoter and determine the maximal promoter activity of the 175-bp fragment of the *SCCA1* gene.

This study also revealed an enhancer region 475-bp to 525-bp upstream of *SCCA1*. It has been reported that intron 1 in sense orientation alone increased 0.3-kb *SCCA1* promoter activity by 2-fold, while that in antisense orientation did not increase it.[12] However, intron 1 in neither sense nor antisense orientation increased *SCCA1* promoter activity in the present study, suggesting that intron 1 is not a major enhancer of *SCCA1*. In stead, we found that the 475-bp to 525-bp fragment increased SCCA1-175 promoter activity by 50% in sense and antisense orientations. The 475-bp to 525-bp fragment thus appears to be a major enhancer region of *SCCA1*. The double and triple enhancers further increased promoter activity compared with the single enhancer in this study. Use of 5 tandem repeats of minimum enhancer element increased promoter activity the most, by 4 times, compared with 1 to 4, 10, or 20 tandem repeats of the

enhancer element. Thus, 5 tandem repeats of minimum enhancer element significantly increased the promoter activity, and thus might enhance the amplification and the anti-tumor effect of replication-competent adenovirus.

SCCA protein, *SCCA1* mRNA, and *SCCA2* mRNA levels and SCCA1-175 promoter activity in squamous cell carcinoma cells were significantly higher than those in normal keratinocytes, which were in turn significantly higher than those in normal non-keratinocyte and adenocarcinoma cells. Although comparisons of *SCCA* gene expression and promoter activity among squamous cell carcinoma cells, normal keratinocytes and adenocarcinoma cells were reported previously,[8, 10] comparisons among squamous cell carcinoma cells, normal keratinocytes and normal non-keratinocyte cells had not previously performed. SCCA1-175 and 525x5-SCCA1-175 promoter activities in normal keratinocytes were significantly higher than those in normal non-keratinocyte cells, and oncolytic adenovirus AdE3-*SCCA1* significantly suppressed *in vitro* growth of normal keratinocyte cells compared with that of normal non-keratinocyte cells. These findings suggest that the *SCCA1* promoter might also be useful for gene therapy for skin disease other than cancer.

The *SCCA1* to *SCCA2* mRNA ratio in squamous cell carcinoma cells was significantly decreased compared with that in the other cells examined, since the relative increase in *SCCA2* mRNA was significantly higher than that in *SCCA1* mRNA in squamous cell carcinoma cells compared with other cells. The adenovirus-mediated *SCCA2* promoter-driven proapoptotic gene thus induced selective tumor suppression of squamous cell carcinoma cells compared with adenocarcinoma cells[11] though this effect was not sufficient to induce complete tumor

regression, since *SCCA1* promoter activity is higher than *SCCA2* promoter activity in squamous cell carcinoma cells. We therefore used the *SCCA1* promoter for gene therapy for squamous cell carcinoma cells. Furthermore, although *SCCA1* promoter alone was not sufficient for overexpression of genes, 5 tandem repeats of enhancer increased *SCCA1* promoter activity by 4-fold and increased the selectivity for squamous cell carcinoma by 2-fold compared with that for other cells examined.

Oncolytic adenovirus AdE3-*SCCA1* selectively killed squamous cell carcinoma cells *in vitro* and *in vivo*. Squamous cell carcinoma can occur in many different organs, including the skin, lips, mouth, esophagus, urinary bladder, prostate, lungs, vagina, cervix, and others. Although oncolytic adenovirus vector driven by *SCCA2* promoter[11] has been reported, *SCCA1* promoter-driven oncolytic virotherapy has not been. The present study is the first report of *SCCA1*-specific gene therapy. In clinical trials for squamous cell carcinoma, although adenovirus-*p53* for head and neck cancer,[14] lung cancer[15] and esophageal cancer,[16] and replicative oncolytic adenovirus for head and neck caner[17] have been reported, the results of such clinical trials were clinically insufficient, since adenoviral infection was completely blocked by the production of anti-adenovirus antibody. This finding is identical to those for gene therapy with other viruses. It has been reported that oncolytic virus-infected carrier cells overcome the viral induced humoral immune response, the viral induced cellular immune response kills virus-infected target cancer cells, and GM-CSF augments the antitumor effect of carrier cells.[18] The present study also demonstrated that oncolytic adenovirus-infected A549 carrier cells induced elimination of tumor and adenovirus-*GM-CSF* augmented the antitumor

effect of carrier cells, which induced complete regression of tumor in 90% of mice. Furthermore, second challenge of syngeneic mouse squamous cell carcinoma was completely rejected by a specific anti-tumor response, also suggesting that the systemic tumor immune response induced by carrier cell treatment may cure not only the carrier cells-injected local tumor but also the non-injected metastatic tumor. In conclusion, *SCCA1* promoter-driven oncolytic adenovirus-infected carrier cells may cure human squamous cell carcinoma of the head and neck, skin, esophagus, lung, cervix, and other organs and human clinical trials by these squamous cell carcinoma-specific carrier cells should be possible in the near future.

Materials and methods

Cell lines and culture conditions

Human non-small cell lung cancer A549, cervical squamous cell carcinoma SKGIIIa and gastric cancer AGS cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Human ovarian carcinoma HEY and 2774 cells from Dr. G. Mills, murine squamous cell carcinoma SCC7 cells from Dr. L. Milas, and human non-small cell lung cancer H1299 cells from Dr. J. A. Roth were obtained (The University of Texas, M.D. Anderson Cancer Center, TX). Normal human keratinocyte SV, HPK, NHK and K42 cells and normal human fibroblast F27 cells were established by Dr. K. Hashimoto, Ehime University, Japan. Normal human fibroblast NF and ovarian fibroblast NOE cells were established in our laboratory. Human umbilical vein endothelial HUVEC cells was obtained from Cambrex Bio Science Walkersville Inc. (Walkersville, MD). Human cervical squamous cell carcinoma HT-III, C4I, C4II, and CaSki cells, and human cervical adenocarcinoma HeLa cells were obtained from the American Type Culture Collection (Rockville, MD).

Cells were maintained in a humidified 5% CO_2/95% air incubator at 37°C. All cell lines except NHK, K42, and HUVEC cells were grown in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum. NHK and K42 cells were grown in MCDB153 (Nissui Co., Tokyo, Japan) with bovine hypothalamus extract. HUVEC cells was grown in EBM-2 (Cambrex, MD)

Construction of the AdE3-SCCA1 vector

The pXC1 plasmid has adenovirus 5 sequences from nt 22 to nt 5790 containing the *E1* gene (Microbix Biosystems Inc., Toronto, Canada). A unique *Age*I site was introduced at nucleotide position 404 after deletion between nt 404 and nt 552 to generate the plasmid pXC1-404-*Age*I. The *SCCA1* promoter was ligated to pXC1-404-*Age*I plasmid to obtain pXC1-*SCCA1*. To construct the AdE3-*SCCA1* virus, homologous recombination was performed between pXC1-*SCCA1* plasmid and the right hand side of pBHGE3 adenovirus DNA containing the *E3* region in 293 cells by a standard technique.[19] To construct the wild type adenovirus AdE3, homologous recombination was performed between pXC1 and pBHGE3 in 293 cells. The replication defective *E1*-deleted Ad5CMV-*LacZ* virus was used as control adenovirus. All viruses were purified with double Cesium Chloride gradients using standard methods, and titered with standard spectrophotometry and plaque assay.[19]

Enzyme-linked immunosorbent assay

Each cell was seeded at 4 x 10^6 cells into 100 mm culture dishes and incubated for 2 days with 10 ml of culture medium. Each cell line was cultured in triplicate dishes. Supernatants were obtained from culture medium in each dish, centrifuged for 5 min at 3000 rpm at room temperature, and assayed to detect SCCA protein. An enzyme-linked immunosorbent assay (ELISA) for SCCA (IMx SCC•DAINAPACK™; Dainabot Co., Tokyo, Japan) was used to evaluate the concentrations of SCCA protein in medium. Medium samples (2 ml) were concentrated ten-fold by centrifugation with a Centricon-10 (Millipore Corp., Bedford, MA). Concentrated samples (200 μl) were applied to an automatic assay apparatus (IMx analyzer;

Dainabot). The lower limit of detection with this ELISA system was 0.01 ng/ml. This assay system could detect both SCCA1 and SCCA2 proteins. The mean values of samples were determined in triplicates. All experiments were performed at least three times and gave similar results.

Real-time quantitative RT-PCR

One hundred ng RNA samples were used in RT and real-time PCR for RNA expression studies. A reverse transcriptase and real-time PCR reaction was carried out with the ABI prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) in a total volume of 50 µl that contained TaqMan one step RT-PCR master mix (Applied Biosystems), 0.3 µM of each forward and reverse primer, and 0.21 µM of MGB probe. The forward and reverse primer and MGB probe were respectively 5'-CCACCGCTGTAGTAGGATTCG-3', 5'-GGAAAGGGTGATTACAATGGAACTC-3' and 5'-ATCATCACCTACTTCAAC-3' for *SCCA1* and 5'-CATGACCTGGAGCCACGG-3', 5'-CCCTCCTCAGTGACCTCCAC-3' and 5'-CTCTCAGTATCTAAAGTCCTAC-3' for *SCCA2*. The reaction was performed with the following thermal cycling method: 30 min at 48°C for reverse transcription, 5 min at 95°C for AmpliTaq Gold activation, 15s at 95°C and 60 s at 60°C for 40 cycles. *GAPDH* was chosen as a housekeeping gene to be tested as an endogenous control.

Assay for promoter activity

SCCA1 and *SCCA2* promoter fragments were inserted into the luciferase reporter vector PicaGene Basic, a promoterless and enhancerless vector (Toyo Ink MFG Co., Tokyo, Japan). The sequence of each insert was confirmed by an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Constructs containing *SCCA1* and *SCCA2* promoter sequences were fused to the *Luciferase* gene which were transfected into cells in the presence of Lipofectamine transfection reagent (Invitrogen, Carisbad, CA), according to the protocol recommended by the manufacturer. Briefly, 1 x 10^5 cells seeded in a 12-well culture dish were exposed to transfection mixtures containing 1 μg of luciferase reporter plasmids and 0.2 μg of p*renilla luciferase-herpes simplex virus thymidine kinase promoter* control vector (Promega, Madison, WI) at 37°C for 48 h. Dual luciferase assays were performed according to the manufacturer's protocol (Promega).

Cell count assay

Cells were plated at a density of 5 x 10^3 cells/well in 12-well plates. Cells were infected with AdE3-*SCCA1* or AdE3. Culture medium alone was used as a mock infection control. After 15 days, cells were harvested and counted to determine the 50% growth inhibitory concentration (IC50).

***Inhibition of subcutaneous tumor growth* in vivo**

To determine inhibition of xenograftic subcutaneous tumor growth, AdE3-*SCCA1* was injected into subcutaneous tumors in female nude (*nu/nu*) mice (CLEA Japan Inc., Tokyo,

Japan). In brief, 1 x 10^7 HT-III or H1299 cells in 100 µl of RPMI were injected into the left posterior flank of each mouse through an insulin syringe with a 27 1/2-gauge needle. Ten animals were used for each group. After 25 days, tumors with a diameter of 5 to 8 mm were established. Then 100 µl of AdE3 (1 x 10^{10} PFU), AdE3-*SCCA1* (1 x 10^{10} PFU), Ad5CMV-*LacZ* (1 x 10^{10} PFU), or medium alone was injected intratumorally on days 0, 1, 2, 3, 4, and 5. The tumors were measured every 5 days with calipers in two perpendicular diameters. Tumor volume was calculated by assuming a spherical shape, with the average tumor diameter calculated as the square root of the product of cross-sectional diameters.

To determine inhibition of syngeneic subcutaneous tumor growth, murine SCC7 cells (1 x 10^6) were injected into the left posterior flank of female C3H mice (CLEA) and AdE3-*SCCA1* was injected into subcutaneous tumors. Ten animals were used for each group. Medium alone, AdE3-*SCCA1* (1 x 10^{10} PFU), or A549 carrier cells (5 x 10^6) infected with AdE3-*SCCA1* at 200 MOI and A549 carrier cells (5 x 10^6) infected with AdE3-*SCCA1* at 200 MOI and AxCA*mGM-CSF* at 10 MOI were injected into the tumors 5 to 8 mm in diameter on days 0, 1, and 2. Mice were preimmunized with Ad-*LacZ* (1 x 10^{10} PFU) on day -21.

Animal studies have been approved by the Ehime University Review Board.

Statistical analysis

Values are the mean ± S.D., and were examined with the unpaired *t*-test, Welch test, and regression analysis. Survival data were plotted on Kaplan-Meier curves, and examined with the log-rank test using the LIFETEST procedure. Findings of $P < 0.05$ were considered significant.

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19. Hamada K, Kohno S, Iwamoto M, Yokota H, Okada M, Tagawa M *et al*. Identification of the human IAI.3B promoter element and its use in the construction of a replication-selective adenovirus for ovarian cancer therapy. *Cancer Res* 2003; **63**: 2506-2512.

Figures

Figure 1. SCCA protein levels determined by ELISA in cervical squamous cell carcinoma, normal keratinocyte, normal non-keratinocyte and adenocarcinoma cells.

Figure 2. *SCCA1* and *SCCA2* mRNA levels determined by real time RT-PCR in cervical squamous cell carcinoma, normal keratinocyte, normal non-keratinocyte and adenocarcinoma cells.

Figure 3. (**a**), A schematic representation of *SCCA1* reporter plasmids. 5'-truncated fragments of the promoter region upstream from the *SCCA1* gene were inserted into luciferase (LUC) reporter vector in sense orientation. Arrow indicates the transcription start site. Numbers indicate the number of bases upstream (-) or downstream (+) from the transcription start site. The name of each reporter construct was assigned according to the 5'-end nucleotide numbers of inserted promoter sequences, upstream of the transcription start site. (**b**), Transcriptional activity of *SCCA1* promoter in HT-III cell line, and identification of core promoter region. *Bars*, SD.

Figure 4. (**a**), A schematic representation of *SCCA1* reporter plasmids with tandem repeats of enhancer region. The intron 1 region or proximal promoter region from -525-bp to -475-bp in sense or antisense orientation was inserted into upstream of the 175-bp region of

SCCA1 luciferase promoter plasmid. Tandem repeats of proximal promoter region from -525-bp to -475-bp were inserted into upstream of the 175-bp region of *SCCA1* luciferase promoter plasmid. (**b**), Luciferase activity of each reporter plasmid was examined in cervical squamous cell carcinoma HT-III. The plasmid (pGV-control) driven by SV40 enhancer/promoter was used as a positive control and pGV-Basic without enhancer/promoter as a negative control. *Bars*, SD. (**c**), Luciferase activity of each reporter plasmid was examined in cervical squamous cell carcinoma HT-III and adenocarcinoma H1299 cells. The plasmid (pGV-control) driven by SV40 enhancer/promoter was used as a positive control and pGV-Basic without enhancer/promoter as a negative control. Luciferase activity in each plasmid was plotted as the ratio to the positive control plasmid (pGV-control). *Bars*, SD. (**d**), Luciferase activities of reporter plasmids SCCA1-175 and SCCA1-175 inserted with 5 tandem repeats from -525 to -475 bp were examined in cervical squamous cell carcinoma, normal keratinocyte, normal non-keratinocyte and adenocarcinoma cells. The plasmid (pGV-control) driven by SV40 enhancer/promoter was used as a positive control and luciferase activity in each cell line was plotted as the ratio to the positive control plasmid. *Bars*, SD.

Figure 5. (**a**), A schematic representation of oncolytic adenovirus AdE3-*SCCA1*. SCCA1-175 with 5 tandem repeats from -525 to -475 bp was replaced with 404 to 551 bp of *E1A* promoter region. (**b**), The growth-inhibitory effects (IC50) of oncolytic adenovirus AdE3-

SCCA1 and wild-type adenovirus AdE3 in cervical squamous cell carcinoma, normal keratinocyte, normal non-keratinocyte and adenocarcinoma cell lines. *Bars,* SD.

Figure 6. (**a**), The antitumor effect of AdE3-*SCCA1* in subcutaneous cervical squamous cell carcinoma HT-III cell tumors in nude mice. (**b**), The antitumor effect of AdE3-*SCCA1* against subcutaneous adenocarcinoma H1299 cell tumors in nude mice. (**c**), The antitumor effects of AdE3-*SCCA1* against subcutaneous SCC7 tumors established in C3H mice after immunization. RPMI medium, AdE3-*SCCA1,* A549 carrier cells infected with AdE3-*SCCA1,* and A549 carrier cells infected with AdE3-*SCCA1* and AxCA*mGM-CSF* were injected into each tumor.

Fig. 1

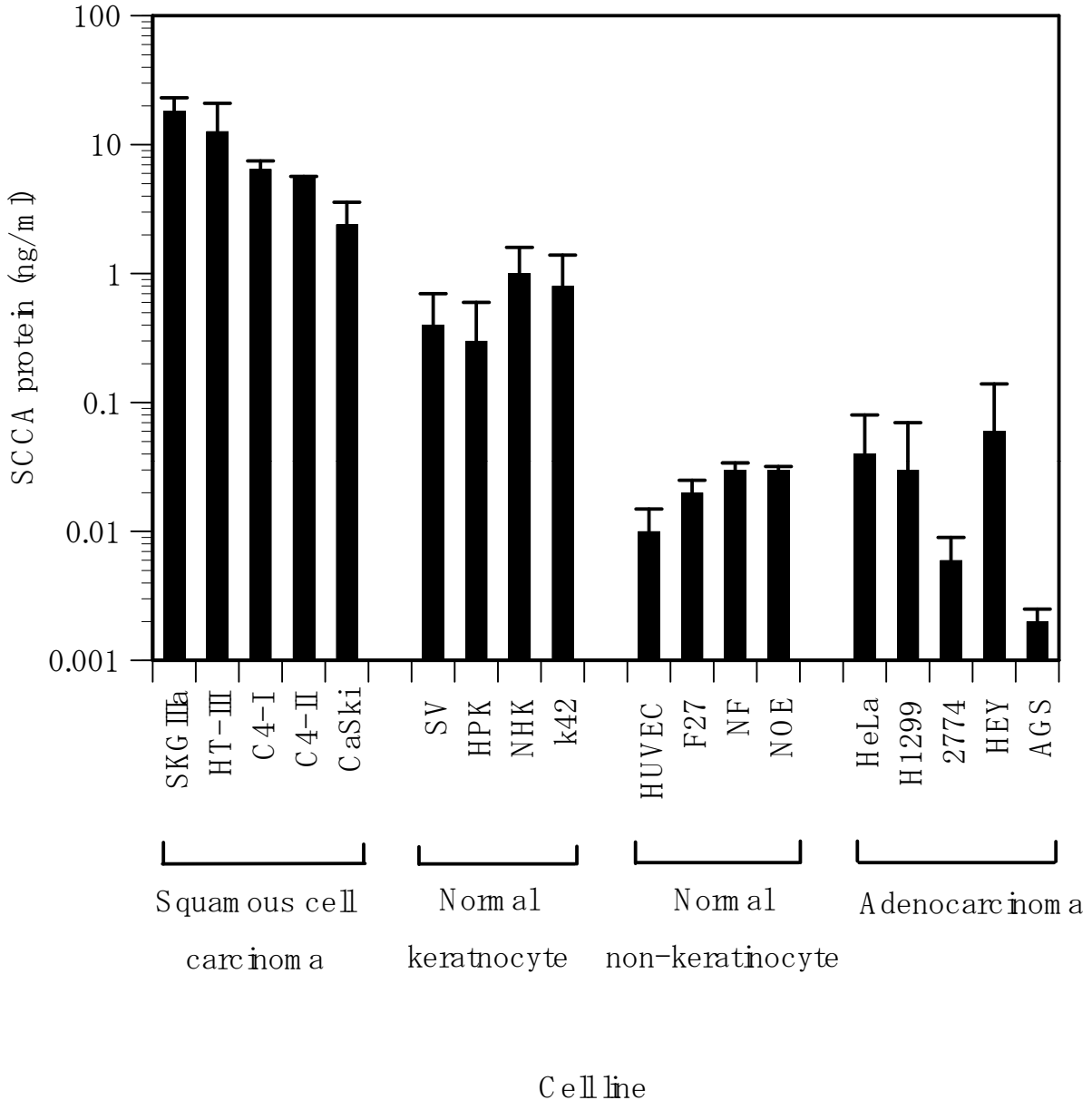

100
10
1
0.1
0.01
0.001
SCCA protein (ng/ml)
SKG IIIa
HT-III
C4-I
C4-II
CaSki
SV
HPK
NHK
k42
HUVEC
F27
NF
NOE
HeLa
H1299
2774
HEY
AGS
Squamous cell carcinoma
Normal keratnocyte
Normal non-keratinocyte
Adenocarcinoma
Cell line

Fig. 2

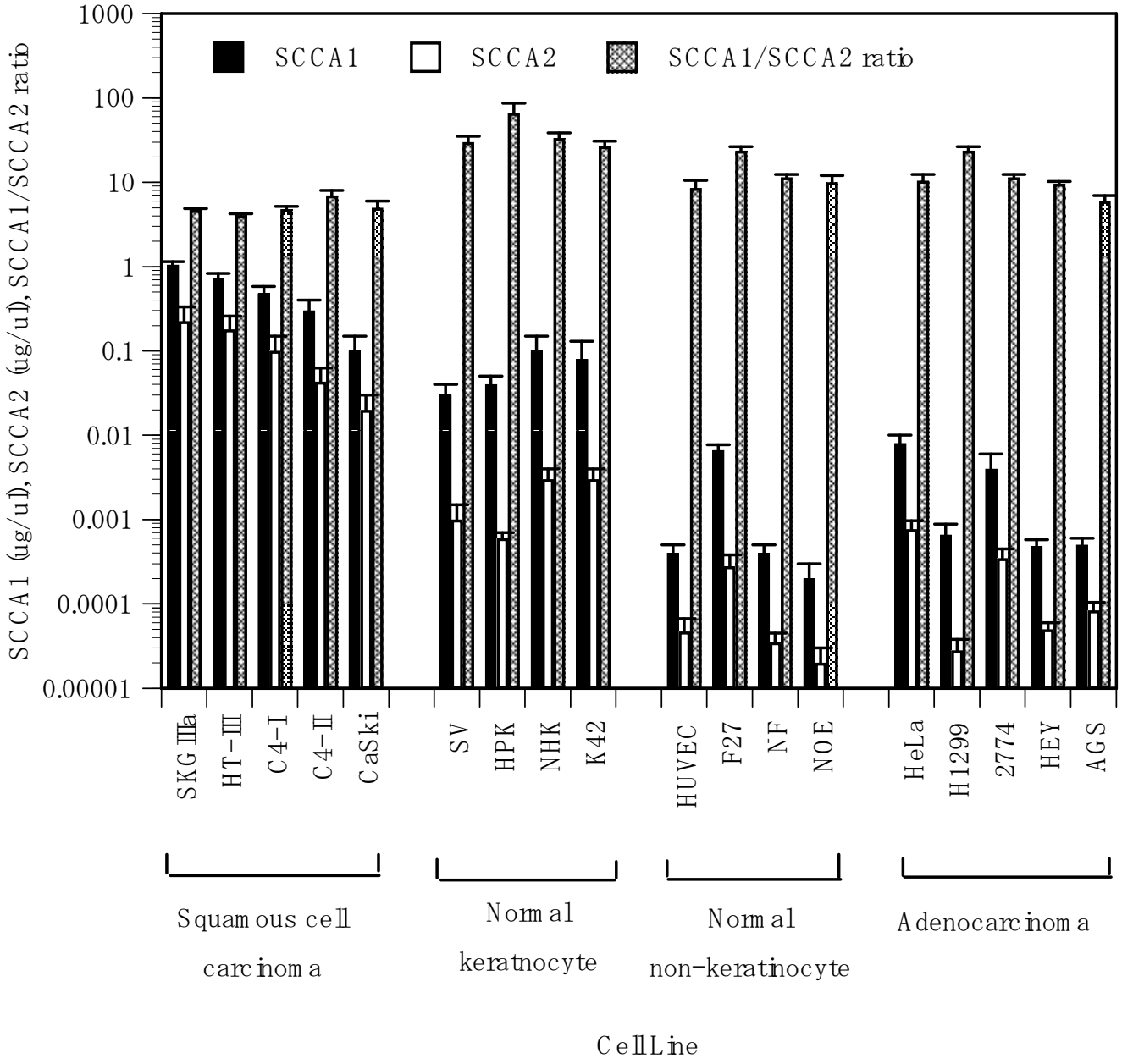

SCCA1
SCCA2
SCCA1/SCCA2 ratio
SCCA1 (ug/ul), SCCA2 (ug/ul), SCCA1/SCCA2 ratio
1000
100
10
1
0.1
0.01
0.001
0.0001
0.00001
SKG IIIa
HT-III
C4-I
C4-II
CaSki
SV
HPK
NHK
K42
HUVEC
F27
NF
NOE
HeLa
H1299
2774
HEY
AGS
Squamous cell carcinoma
Normal keratnocyte
Normal non-keratinocyte
Adenocarcinoma
CellLine

Fig. 3a

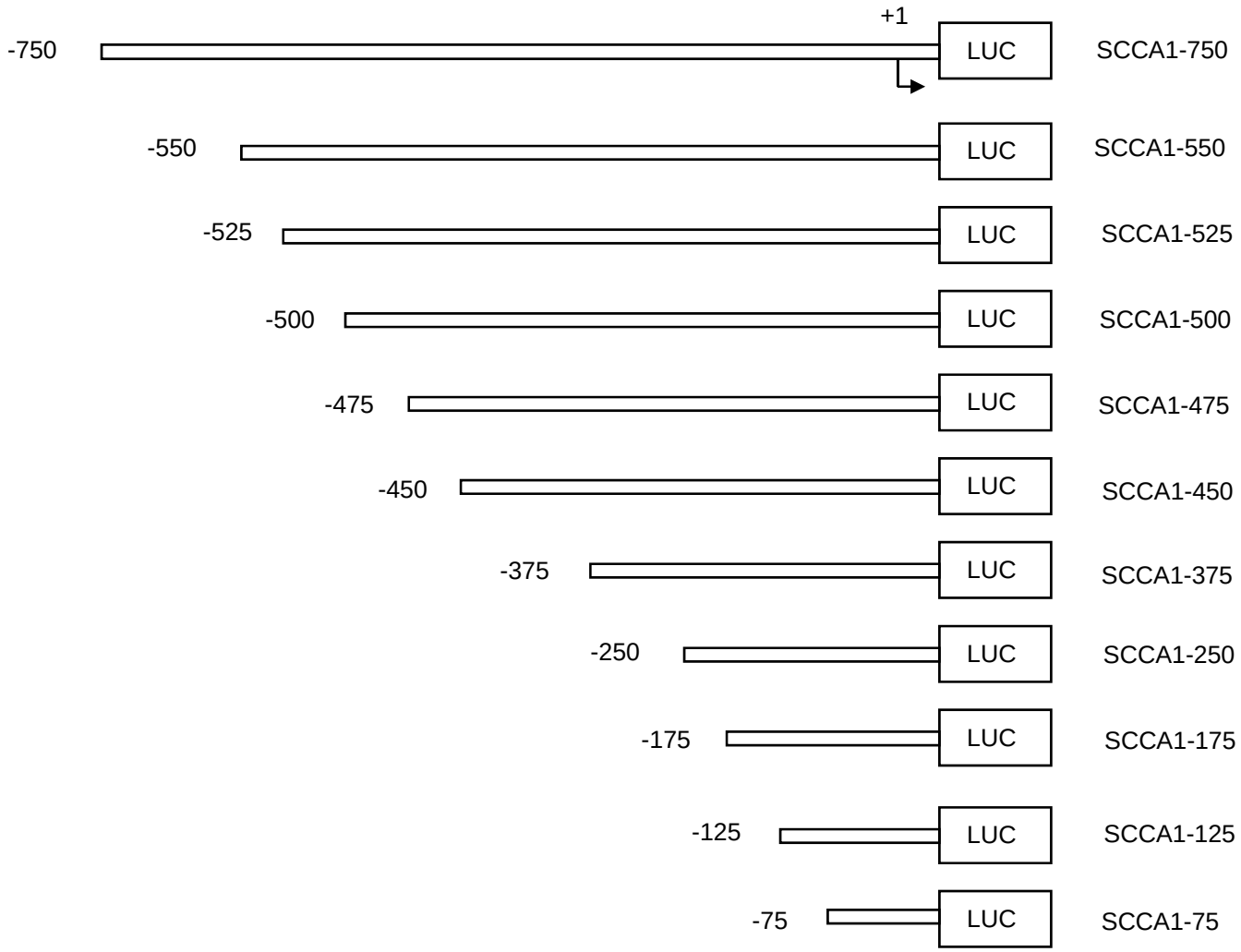

+1
-750
LUC
SCCA1-750
-550
LUC
SCCA1-550
-525
LUC
SCCA1-525
-500
LUC
SCCA1-500
-475
LUC
SCCA1-475
-450
LUC
SCCA1-450
-375
LUC
SCCA1-375
-250
LUC
SCCA1-250
-175
LUC
SCCA1-175
-125
LUC
SCCA1-125
-75
LUC
SCCA1-75

Fig. 3b

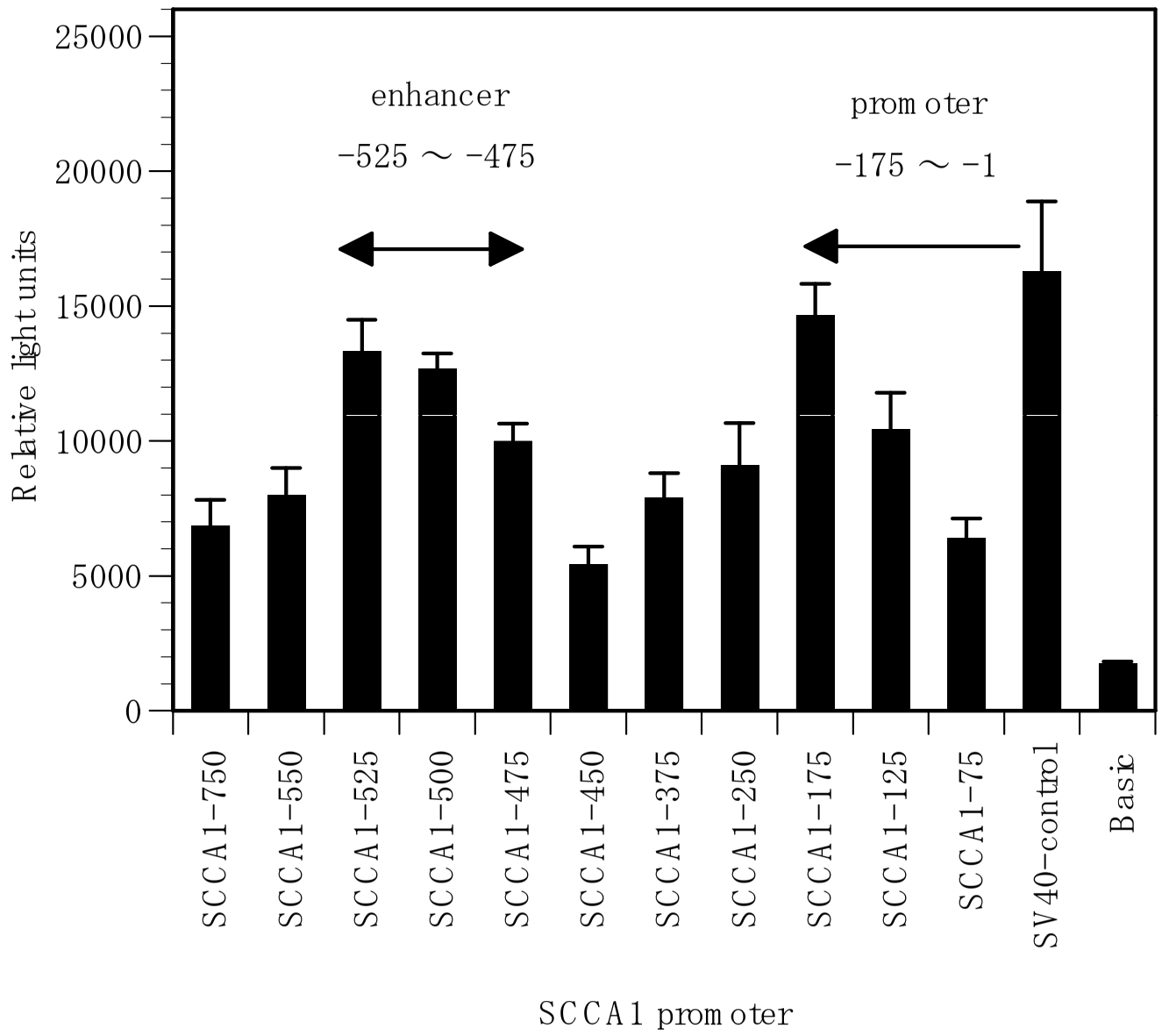

25000
20000
15000
10000
5000
0
Relative light units
enhancer
-525 ~ -475
promoter
-175 ~ -1
SCCA1-750
SCCA1-550
SCCA1-525
SCCA1-500
SCCA1-475
SCCA1-450
SCCA1-375
SCCA1-250
SCCA1-175
SCCA1-125
SCCA1-75
SV40-control
Basic
SCCA1 promoter

Fig. 4a

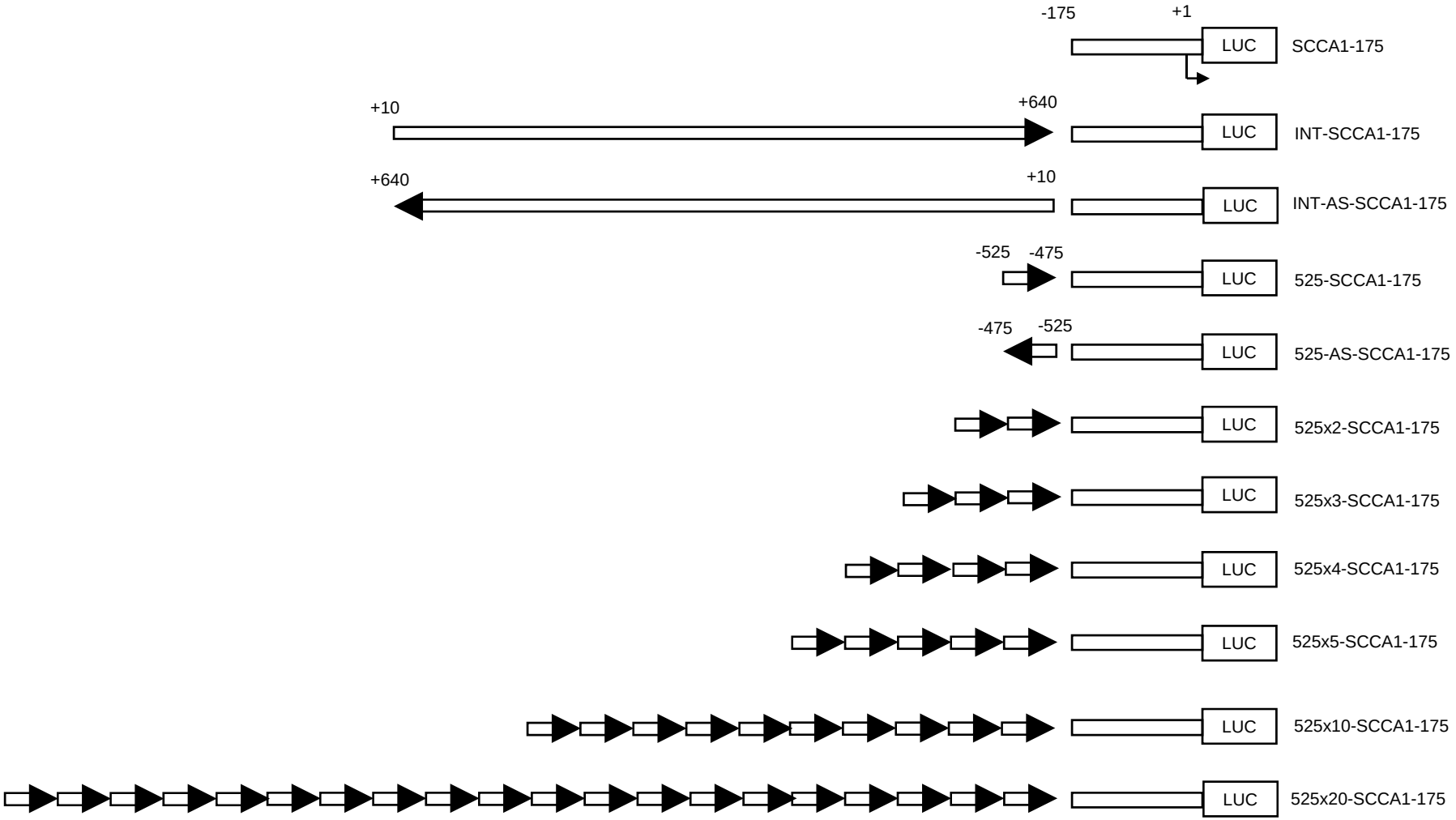

-175
+1
LUC
SCCA1-175
+10
+640
LUC
INT-SCCA1-175
+640
+10
LUC
INT-AS-SCCA1-175
-525
-475
LUC
525-SCCA1-175
-475
-525
LUC
525-AS-SCCA1-175
LUC
525x2-SCCA1-175
LUC
525x3-SCCA1-175
LUC
525x4-SCCA1-175
LUC
525x5-SCCA1-175
LUC
525x10-SCCA1-175
LUC
525x20-SCCA1-175

Fig. 4b

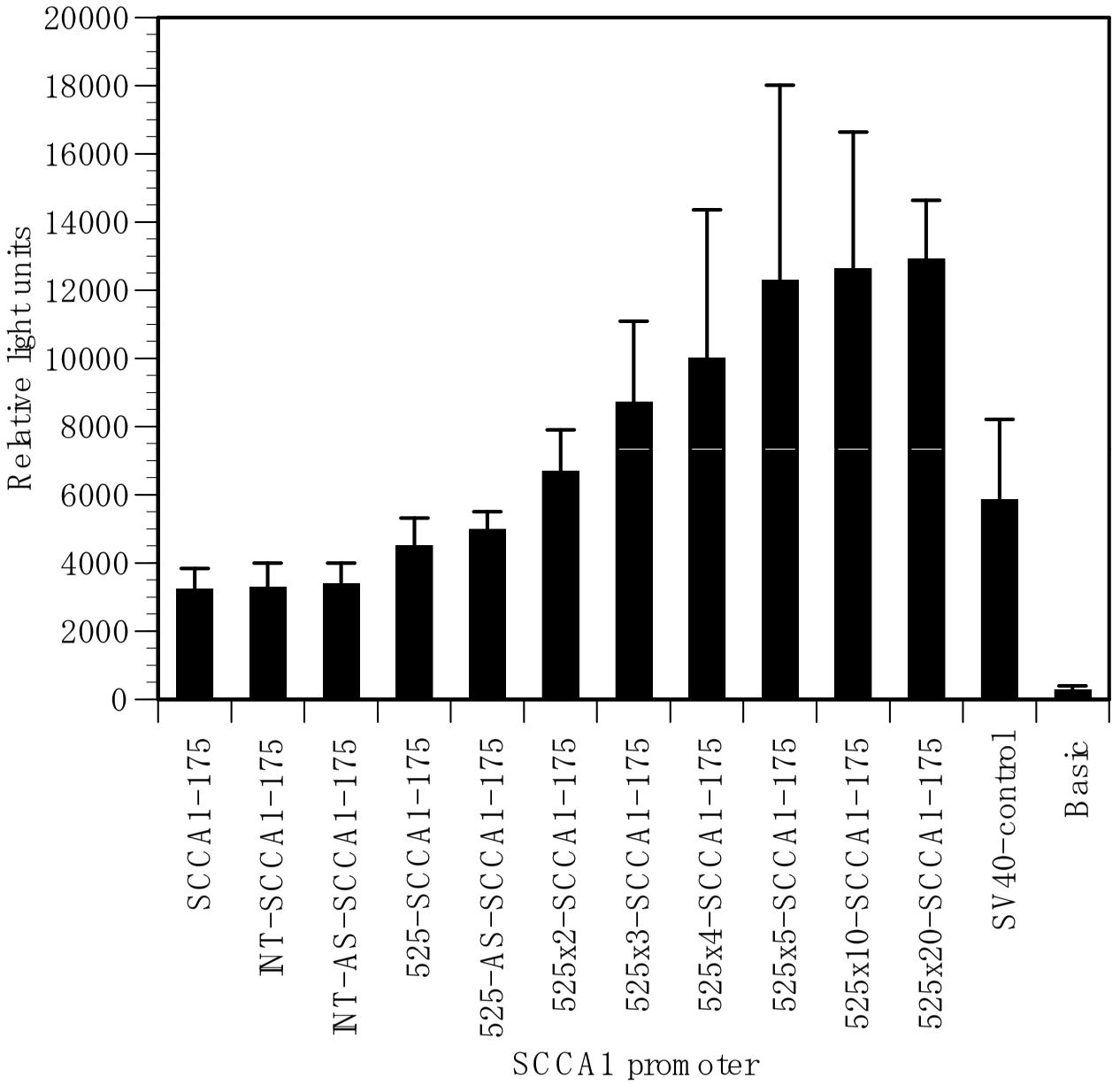

20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Relative light units
SCCA1-175
NT-SCCA1-175
NT-AS-SCCA1-175
525-SCCA1-175
525-AS-SCCA1-175
525x2-SCCA1-175
525x3-SCCA1-175
525x4-SCCA1-175
525x5-SCCA1-175
525x10-SCCA1-175
525x20-SCCA1-175
SV40-control
Basic
SCCA1 promoter

Fig. 4c

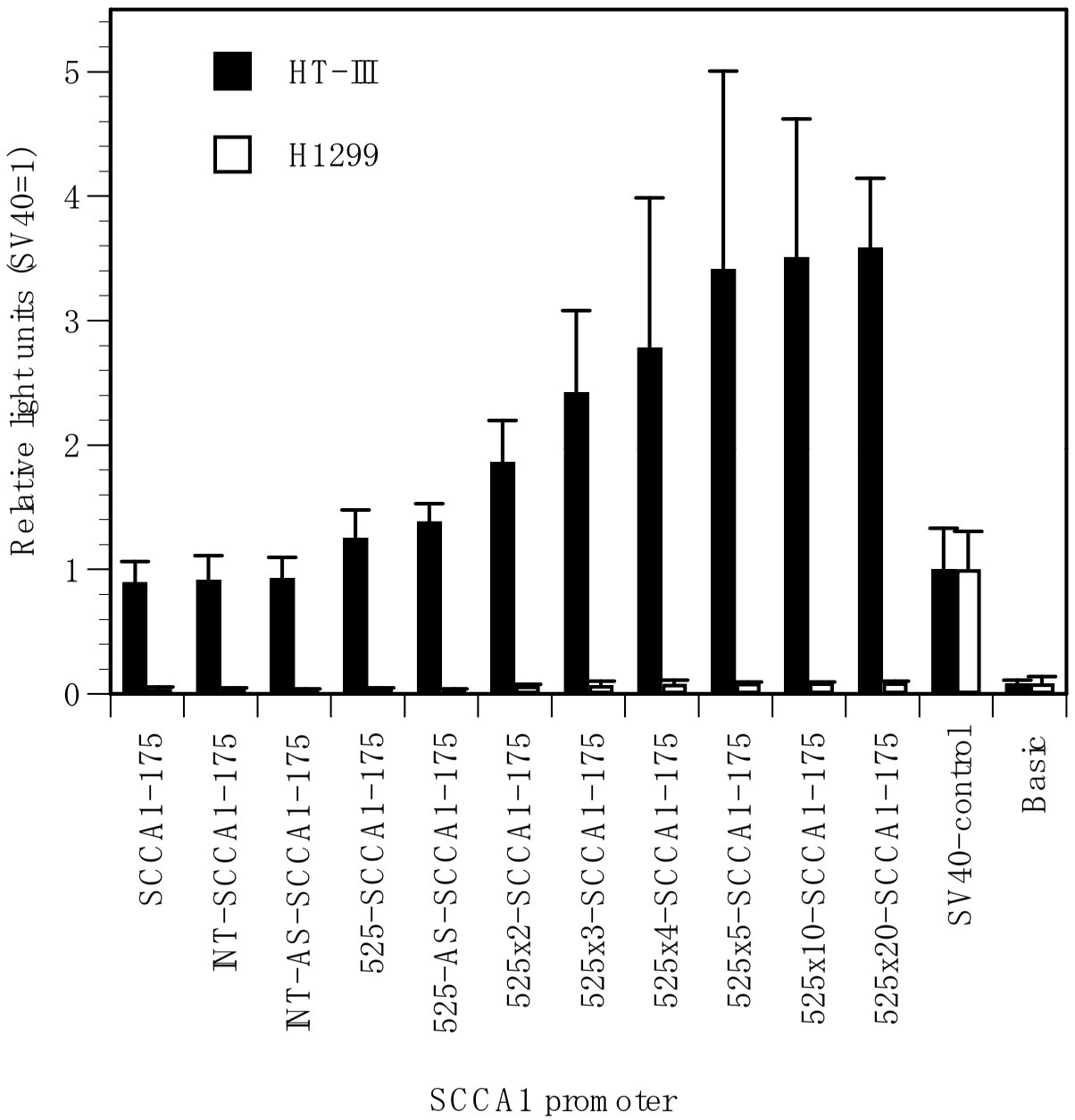
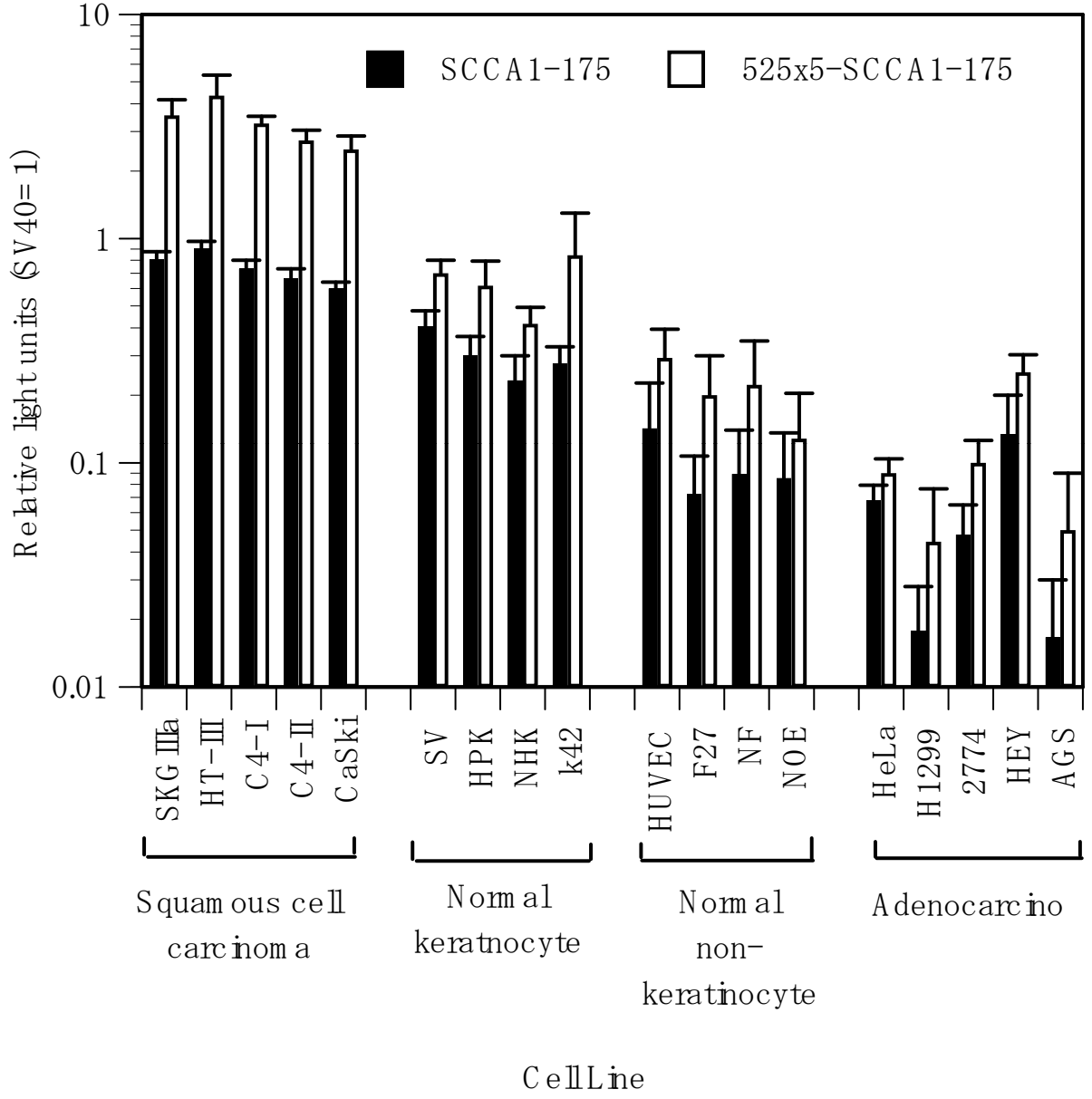

HT-III
H1299
Relative light units (SV40=1)
0
1
2
3
4
5
SCCA1-175
NT-SCCA1-175
NT-AS-SCCA1-175
525-SCCA1-175
525-AS-SCCA1-175
525x2-SCCA1-175
525x3-SCCA1-175
525x4-SCCA1-175
525x5-SCCA1-175
525x10-SCCA1-175
525x20-SCCA1-175
SV40-control
Basic
SCCA1 promoter

Fig. 4d


10
1
0.1
0.01
Relative light units (SV40=1)
SCCA1-175
525x5-SCCA1-175
SKG IIIa
HT-III
C4-I
C4-II
CaSki
SV
HPK
NHK
k42
HUVEC
F27
NF
NOE
HeLa
H1299
2774
HEY
AGS
Squamous cell carcinoma
Normal keratnocyte
Normal non-keratinocyte
Adenocarcino
CellLine

F i g . 5a

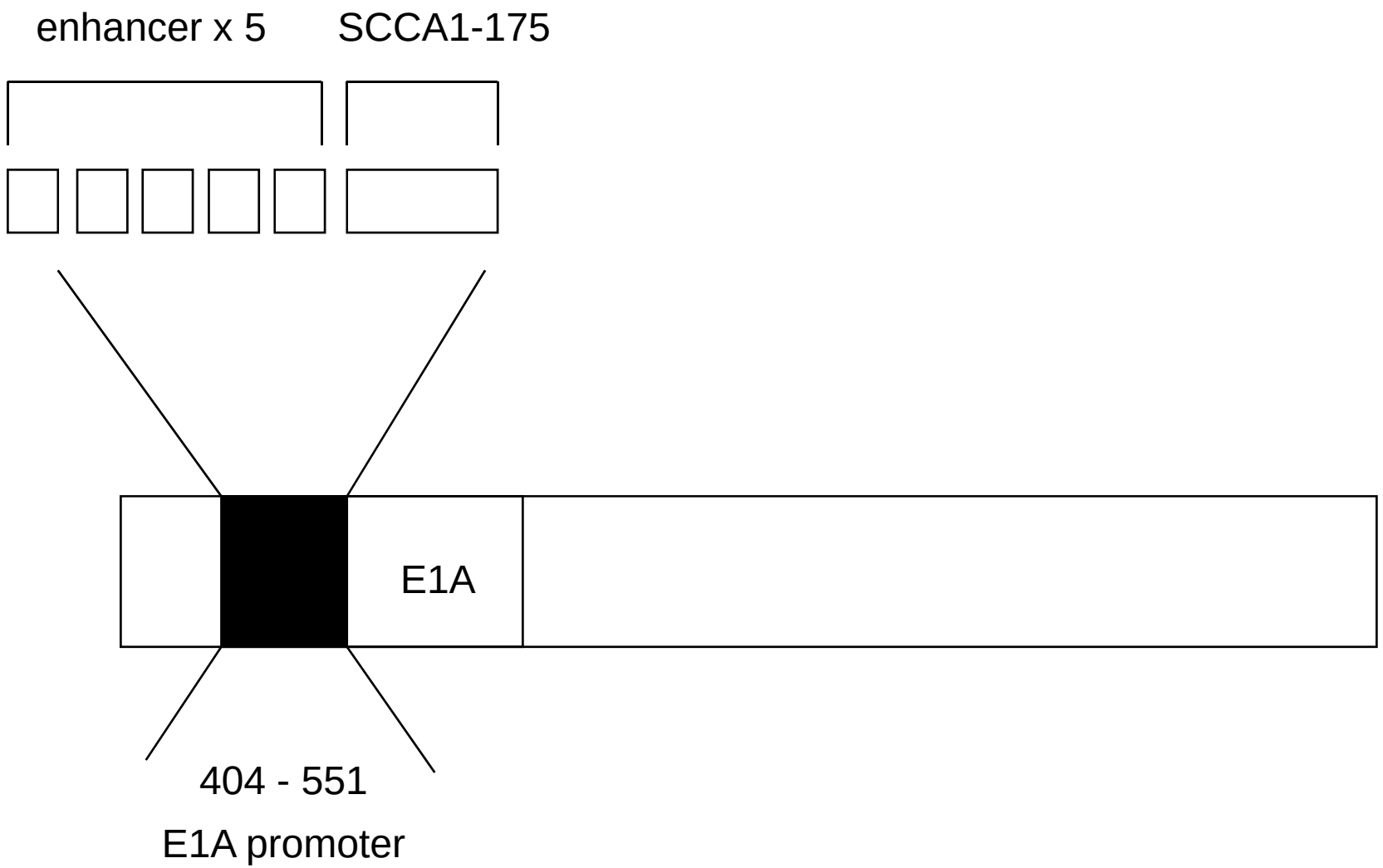


Fig. 5b

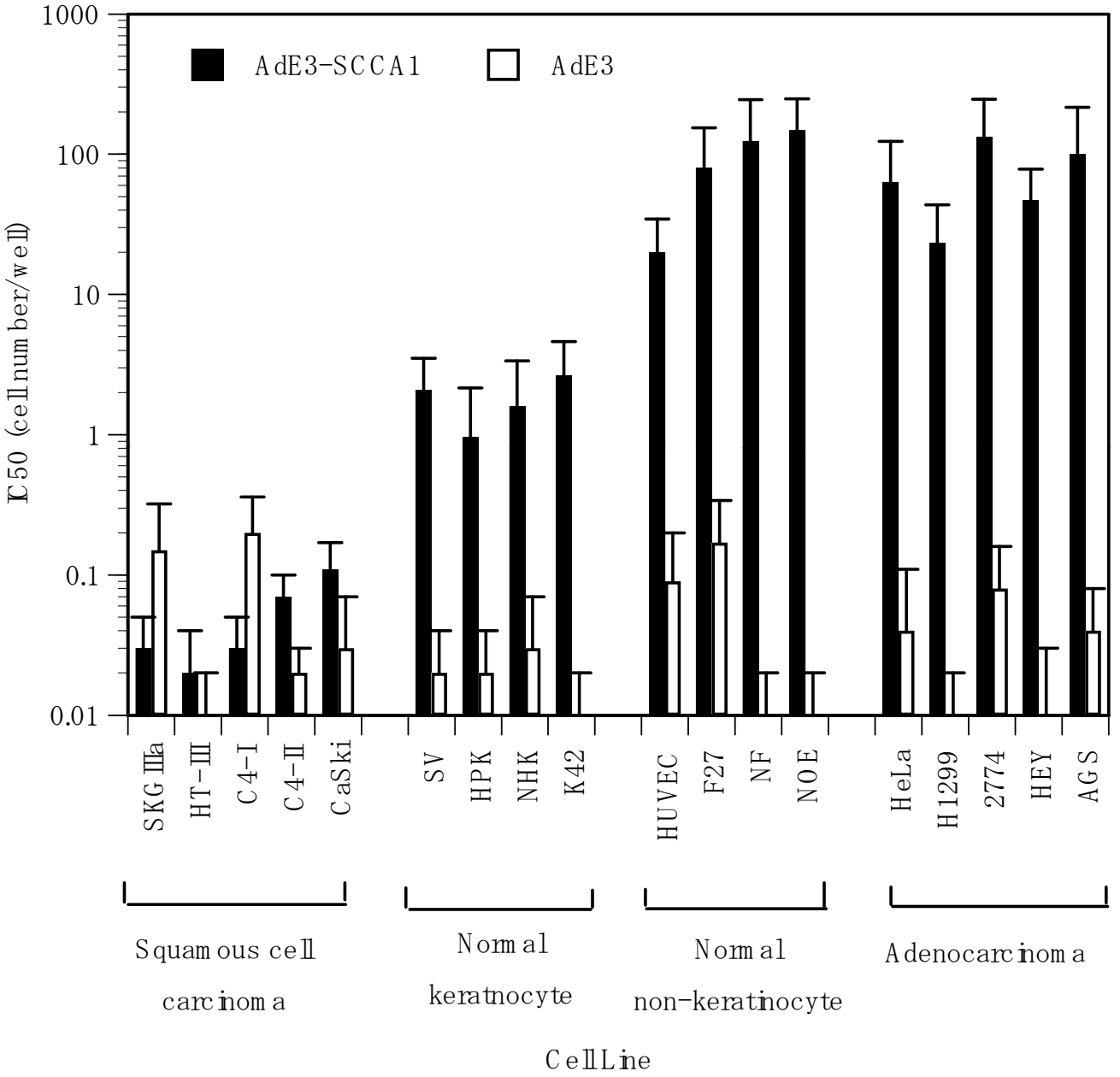

1000
100
10
1
0.1
0.01
IC50 (cell number/well)
AdE3-SCCA1
AdE3
SKG IIIa
HT-III
C4-I
C4-II
CaSki
SV
HPK
NHK
K42
HUVEC
F27
NF
NOE
HeLa
H1299
2774
HEY
AGS
Squamous cell carcinoma
Normal keratnocyte
Normal non-keratinocyte
Adenocarcinoma
CellLine

Fig. 6a

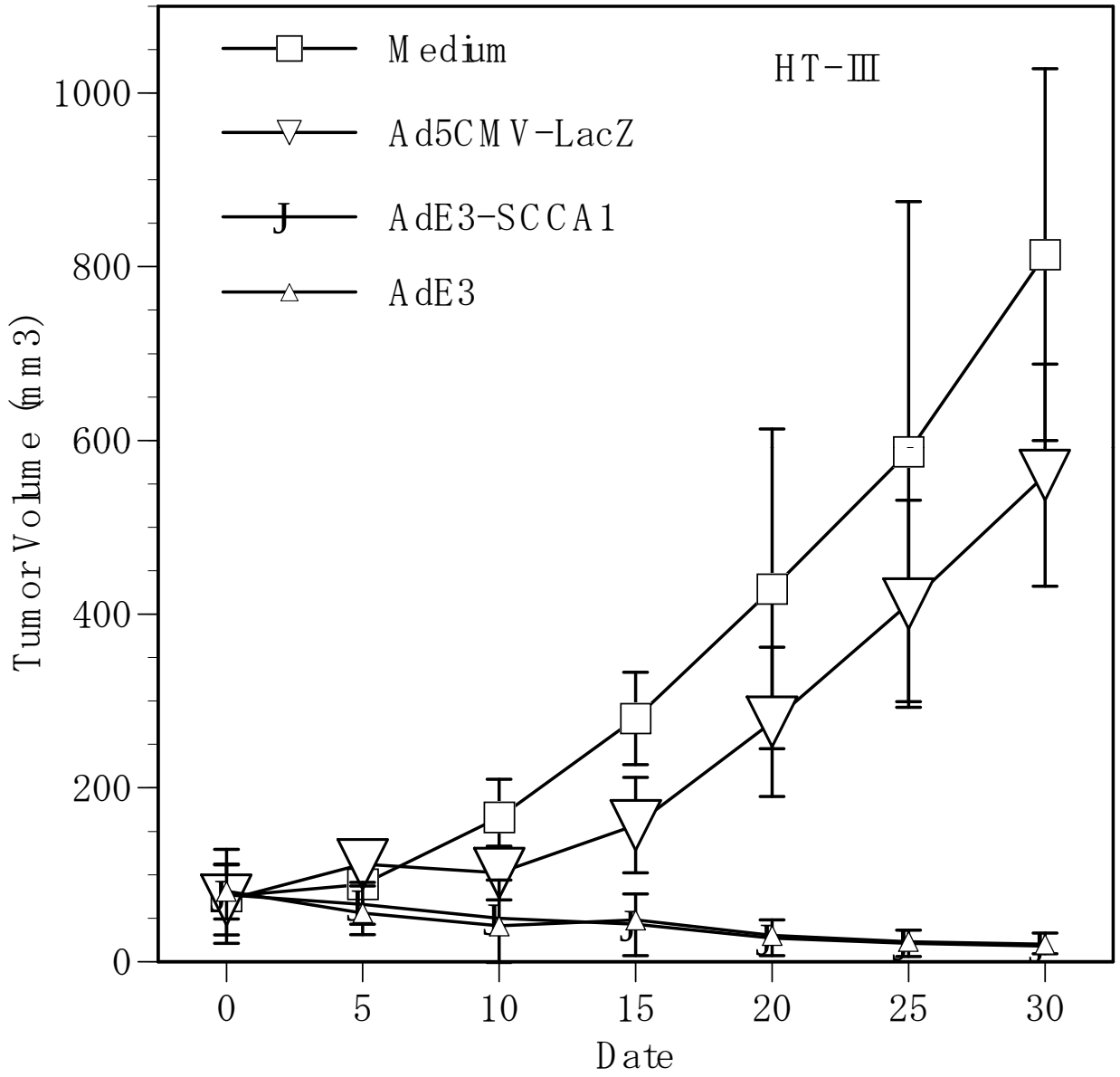

Medium
Ad5CMV-LacZ
AdE3-SCCA1
AdE3
HT-Ⅲ
Tumor Volume (mm3)
1000
800
600
400
200
0
0
5
10
15
20
25
30
Date

Fig. 6b

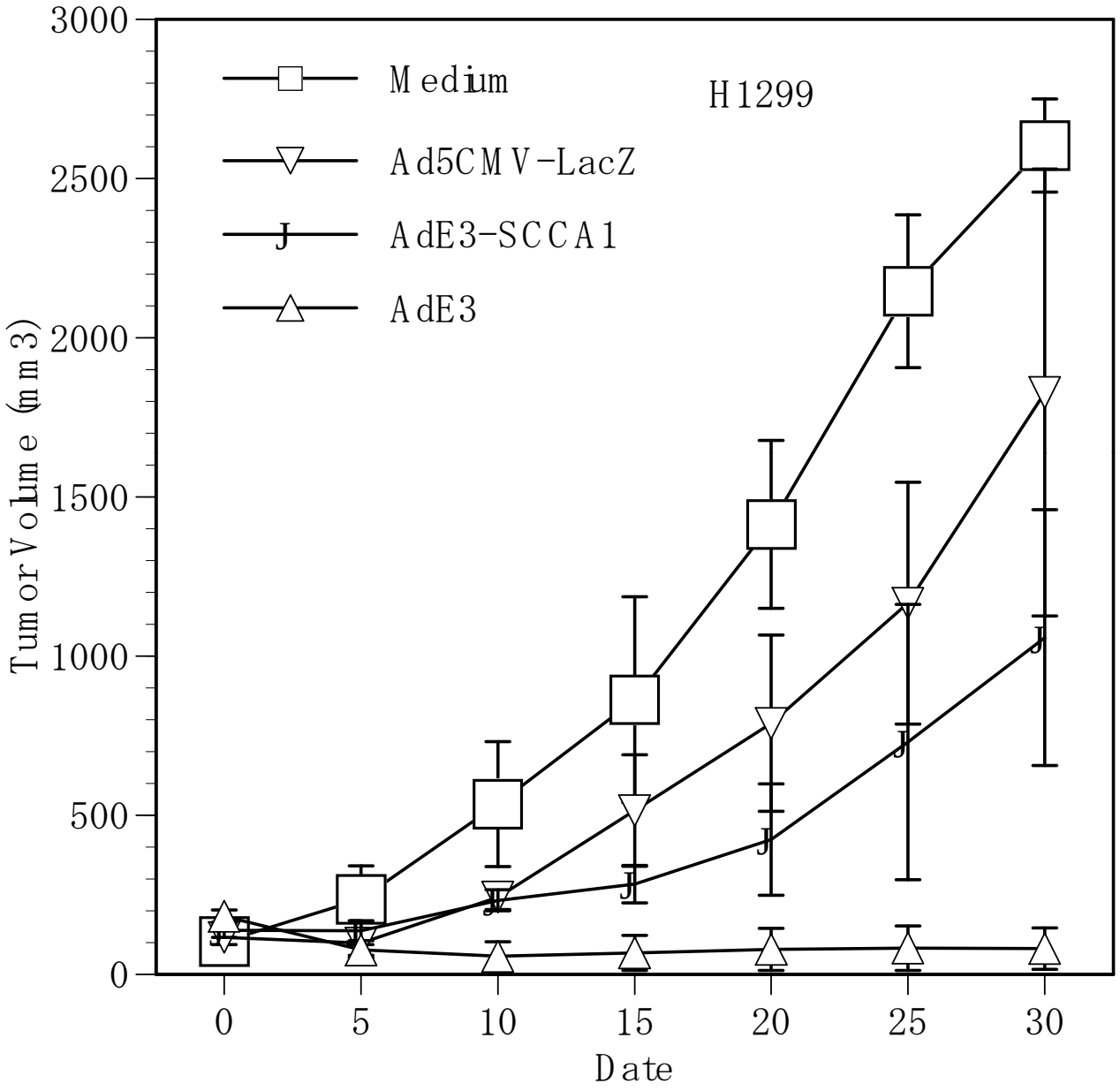
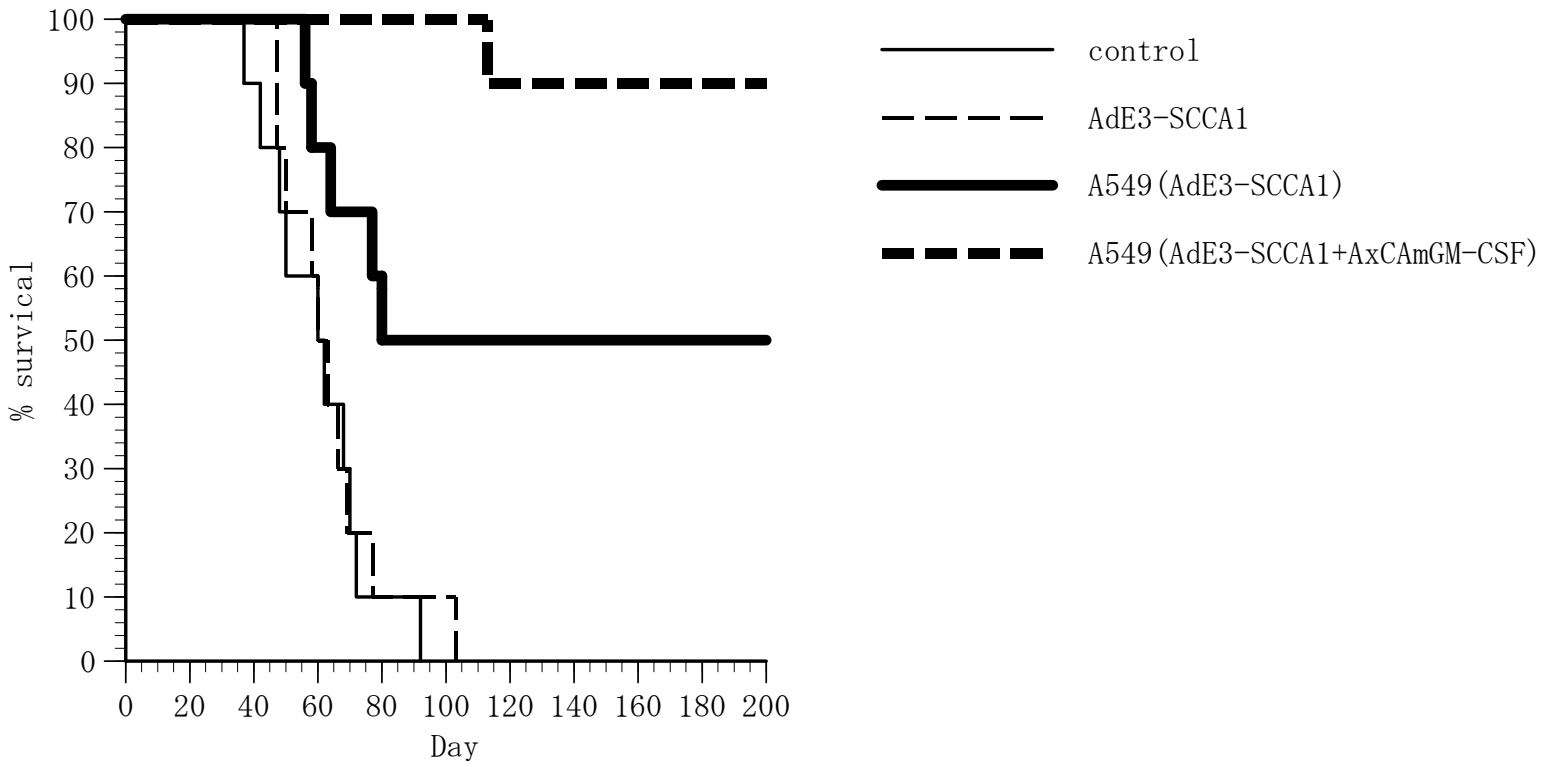

3000
2500
2000
1500
1000
500
0
Tumor Volume (mm3)
0
5
10
15
20
25
30
Date
Medium
Ad5CMV-LacZ
AdE3-SCCA1
AdE3
H1299

Fig. 6c


100
90
80
70
60
50
40
30
20
10
0
% survical
0
20
40
60
80
100
120
140
160
180
200
Day
control
AdE3-SCCA1
A549(AdE3-SCCA1)
A549(AdE3-SCCA1+AxCAmGM-CSF)