1	• Title
2	The induction of antigen-specific CTL by in situ Ad-REIC gene therapy
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22	Okayama University and Momotaro-Gene Inc. are applying for patents on the Ad-REIC systems.
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24	Momotaro-Gene Inc.
25	

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1 • Abstract

 $\mathbf{2}$ An adenovirus vector carrying the human REIC/Dkk-3 gene (Ad-REIC) mediates simultaneous 3 induction of cancer-selective apoptosis and augmentation of anti-cancer immunity. In our preclinical and clinical studies, in situ Ad-REIC gene therapy showed remarkable direct and 4 5 indirect anti-tumor effects to realize therapeutic cancer vaccines. We herein aimed to confirm 6 the induction of tumor-associated antigen specific cytotoxic T-lymphocytes (CTLs) by Ad-REIC. 7 Using an OVA, a tumor associated antigen (TAA), expressing E.G7 tumor-bearing mouse model, we investigated the induction and expansion of OVA-specific CTLs responsible for indirect, 8 9 systemic effects of Ad-REIC. The intratumoral administration of Ad-REIC mediated clear 10 anti-tumor effects with the accumulation of OVA-specific CTLs in the tumor tissues and spleen. 11 The CD86-positive dendritic cells (DCs) were up-regulated in the tumor draining lymph nodes 12of Ad-REIC treated mice. In a dual tumor-bearing mouse model in the left and right back, 13Ad-REIC injection in one side significantly suppressed the tumor growth on both sides and 14significant infiltration of OVA-specific CTLs into non-injected tumor was also detected. 15Consequently, in situ Ad-REIC gene therapy is expected to realize a new generation cancer vaccine via anti-cancer immune activation with DC and tumor antigen-specific CTL expansion. 1617

1 • Introduction

 $\mathbf{2}$ The Reduced Expression in Immortalized Cells (REIC) gene is a member of the DKK family 3 genes (hDKK-1,2,3 and 4) with homology to Xenopus laevis Dickkopf gene. REIC/Dkk-3 was 4 originally isolated as an immortalization related gene of normal human fibroblasts and was $\mathbf{5}$ regarded as a tumor suppressor gene (1, 2). The expression of the REIC/Dkk-3 gene is 6 significantly reduced in a wide range of cancer cells (3-6). Although Dkk-3 does not bind with $\overline{7}$ LDL-receptor-related protein 5/6 (LRP5/6) and the precise mechanism by which Dkk-3 8 interferes with Wnt signaling remains an obstacle (7-9), its biological functions in the 9 embryonic development (10, 11) and emerging roles in cancer therapy (2) have been studied 10 with increasing attention. In addition, our previous study showed that REIC/Dkk-3 protein has a 11 cytokine-like effects, responsible for the differentiation of human CD14+monocyte into a novel 12cell type, dendritic cell (DC)-like cell (12, 13).

13An adenovirus vector carrying the human REIC/Dkk-3 gene (Ad-REIC) allowed the forced expression of REIC/Dkk-3, and induced apoptosis in a variety of cancer cells in vitro and in 1415vivo (14-18). The molecular mechanisms of these apoptotic events have been proved to be induced by unfolded protein response in the endoplasmic reticulum (ER stress) with c-Jun 1617N-terminal kinase (JNK) activation (19-21). Interestingly, ER stress mediates the enhanced IL-7 expression in co-infected normal fibroblasts, resulting in the activation of innate immunity 18 involving natural killer (NK) cells (22). Furthermore, the secreted REIC protein with 1920immunomodulatory function creates an optimal environment for the activation of host immune 21cells, inducing cytotoxic T lymphocytes (12, 13, 23).

In the present study, we focus on the antigen-specific CTL responses mediated by *in situ* Ad-REIC gene therapy. Using a model of a tumor cell line carrying specific antigen, ovalbumin (OVA), we investigated *in vivo* antitumor effects via OVA specific immune response generated by Ad-REIC gene therapy.

1 • Results

2 REIC/Dkk-3 induced apoptosis in E.G7 cell lines

3 The specific induction of apoptosis was demonstrated in E.G7 cells by Ad-REIC treatment. 4 We first assessed the *in vitro* apoptotic effects of Ad-REIC in comparison to control vector, 5 Ad-LacZ. E.G7 cells were infected with Ad-LacZ or Ad-REIC at 100 MOI and 500 MOI. We 6 monitored apoptotic cells at 48 h after infection by Hoechst staining. The Ad-REIC vector 7 significantly enhanced the *in vitro* apoptosis induction in comparison to the control vectors 8 transfected at 500 MOI. (Figure. 1a and 1b) At 500 MOI, 41.2 ± 8.64 % of Ad-REIC-infected 9 E.G7 cells were positive for Hoechst staining; in contrast, only 18.4 ± 4.16 % of Ad-LacZ cells 10 were positive (P<0.01). 11 12In vivo Ad-REIC gene therapy suppressed the growth of E.G7 tumors 13We used an E.G7 mouse model to reveal the direct antitumor effects of Ad-REIC gene therapy (Figure 2a). We first evaluated the levels of REIC/Dkk-3 protein expression in E.G7 14tumors. The overexpression of REIC/Dkk-3 was observed after the intratumoral injection of 1516Ad-REIC in E.G7 tumors; this response was not observed in control settings (Figure 2b). We 17next investigated whether Ad-REIC inhibits tumor growth in vivo. The significant suppression 18 of tumor growth was observed from day 10 in the Ad-REIC treated group in comparison to the Ad-LacZ group (P < 0.01) (Figure 2c). 19

20

21 Tumor antigen-specific CTLs were induced by Ad-REIC gene therapy

22 We examined whether tumor antigen-specific CTLs could be induced by Ad-REIC gene

23 therapy. E.G7 tumor bearing mice were intratumorally injected with Ad-REIC or Ad-LacZ. Two

- 24 days after the injection, the mice were sacrificed and examined to determine whether
- 25 OVA-specific CTLs had accumulated in the tumor-infiltrating lymphocytes (TILs).
- 26 OVA-specific CTLs were detected by OVA tetramer composing MHC H-2 Kb + OVA 257-264.

1	As shown in Figure 3a, a higher percentage of OVA tetramer + CD8 + T cells was detected in
2	the Ad-REIC treated TILs in comparison to the control vector injected mice (18.8 % vs. 3.4 %,
3	respectively; $P = 0.02$). We next evaluated the systemic immune response. After injection of the
4	vector, the spleen was harvested and single cell suspensions were prepared. Splenetic cells were
5	cultured with OVA peptide for 7 days. After ex vivo OVA peptide stimulation, OVA-specific
6	CTLs were detected by OVA tetramer. OVA tetramer+ cells were significantly higher in the
7	Ad-REIC treated spleen in comparison to the control mice (24.2 % vs. 1.1 %, respectively;
8	P = 0.04) (Figure 3b). These results showed that Ad-REIC gene therapy significantly induced
9	OVA-specific CTL responses in both the TILs and the lymphoid organs (Figure 3c).

10

11 Induced CTL produce IFN-y.

12To evaluate the production ability of IFN- γ , CD8+ TILs and the tumor draining lymph nodes 13(TDLNs) were cultured with PMA/ionomycin for 4 h. PMA was a useful tool for monitoring the 14capacity for cytokine production of CD8+ T cells (24). IFN- γ producing cells were not observed 15in Ad-LacZ treated TILs and TDLN. The CTLs from Ad-REIC treated mice produced large 16amounts of IFN-y after PMA/ionomycin stimulation (Figure 4a, b). As compared to Ad-LacZ, 17Ad-REIC gene therapy strongly induced IFN-γ production in CD8+ TILs and the TDLNs (TILs: 0.07 ± 0.11 % vs. 2.27 ± 0.63 %, respectively; P = 0.02; TDLN: 0.18 ± 0.07 % vs. 2.34 ± 0.58 %, 1819respectively; P = 0.02) (Figure 4c).

20

21 Characterization of dendritic cells in the tumor draining lymph node

To investigate the dendritic cell (DC) subsets after Ad-REIC treatment, TDLNs were removed and analyzed by flow cytometry. In the TDLNs, there were two subsets: CD11c^{int} MHC class II^{high} subset (mDC) and CD11c^{high} MHC class II^{int} subset (rDC) (25). In the Ad-REIC injection mice, more than 1.0 x 10⁴ cells were mDCs and about 7.0 x 10³ cells were rDCs. On the other hand, the numbers of cells in both DC subsets were <5.0 x 10³ in Ad-LacZ treated mice (Figure 5a). CD103 (+) mDC and CD8 (+) rDC are known to have the ability of cross-presentation
(26-28). Significant amount of induced DCs in Ad-REIC injected mice were found to be subset
with ability of cross-presentation (Figure 5b). More importantly, in Ad-REIC injected TDLNs,
both DC subsets showed higher expression levels of CD86, which is used as an activation
marker, in comparison to Ad-LacZ mice (Figure 5c).

6

7 The systemic antitumor effect could be induced by in-situ Ad-REIC gene therapy

8 In order to assess anti-tumor effects of Ad-REIC gene therapy on distant tumors, we 9 developed a dual tumor-bearing mouse model (Figure 6a). E.G7 cells were injected into the left and right back of a mouse. When the tumor diameter reached 5mm, Ad-REIC was injected into 10 11 the right side of the tumor only. Forty-eight hours after the second injection, the TILs were 12examined to detect OVA-specific CTLs. At 1 week after the second injection, complete tumor 13growth suppression was observed in the Ad-REIC treated group, though the dual tumor grew in 14the Ad-LacZ treated mice (Figure 6a). To evaluate the indirect immune response, TILs were harvested from the non-injection side. As shown in Figure 6b, a significant number of 15OVA-specific CD8+ T cells were detected in non-injected tumor of Ad-REIC treated mice as 1617compared to control Ad-LacZ treated mice (11 % vs. 0.65 %, respectively; P < 0.01) (Figure 6b). In this dual tumor-bearing mouse model, it was confirmed that OVA-specific CTLs derived 18 19from Ad-REIC injected tumor were responsible for anti-tumor effects on non-injected tumor. 20Using this model and anti-CD8 antibody to deplete the T cell functions, we also confirmed that 21the anti-tumor effects were dependent on the CD8+ T lymphocyte, including the CTLs (Figure 226c, d). We also examine anti-tumor effects in the other dual tumor-bearing mouse model of 23prostate cancer. Using human prostate specific antigen (PSA)-expressing RM9 cancer cells, we 24further demonstrated the anti-tumor effects in the dual tumor (Figure 6e).

25

 $\mathbf{6}$

1 • Discussion

 $\mathbf{2}$ The new strategy, based on the concept of simultaneous induction of selective killing of cancer 3 cells and augmentation of antitumor immunity, leads to a new generation of cancer vaccines and 4 it is expected to become a leading standard in the treatment of most solid cancers with gene $\mathbf{5}$ therapy. To realize this concept, oncolytic viruses armed with GM-CSF, such as T-VEC 6 (talimogene laherparepvec, an oncolytic herpes simplex type 1 virus) and Pexa-Vec $\overline{7}$ (pexastimogene devacirepvec; an oncolytic vaccinia poxvirus) have already been successfully 8 developed (29-31). Similarly, we are now developing Ad-REIC as a new therapeutic cancer 9 vaccine using an original multifunctional therapeutic gene of REIC/Dkk-3.

10 In our preclinical and clinical studies on *in situ* gene therapy, indirect, systemic anti-tumor 11 effects induced by Ad-REIC were found to be strong enough to be realized as therapeutic cancer 12vaccines. For instance, in an orthotopic prostate cancer model with pre-established lung 13metastases using RM-9 mouse prostate cancer cells, intraprostatic injection of Ad-REIC significantly suppressed the local tumor growth and pre-established lung metastases, leading to 1415a prolonged mice survival (12, 32). The First-In-Man clinical study, a phase I/IIa study of in situ Ad-REIC gene therapy for prostate cancer (PCa), was initiated at Okayama University in 1617January 2011(33). Two groups of patients were treated at 4 escalating doses: group A consisting of patients with castration-resistant PCa (CRPC) with or without metastasis, and group B 18 consisting of patients with high-risk, localized PCa scheduled to undergo radical prostatectomy. 1920As of November 2014, 8 patients in group A and 18 scheduled patients in group B were treated, 21demonstrating remarkable safety profiles of Ad-REIC. These clinical data with dose-dependent 22decrease in PSA (a tumor marker), immuno-pathological effects in surgical specimens and 23favorable outcomes in biochemical recurrence-free survival after radical prostatectomy in group 24B will be published elsewhere. In group A, dramatic systemic effects induced by in situ gene 25therapy were illustrated in a case of chemotherapy resistant advanced CRPC with bulky lymph 26node metastasis (33). According to these preclinical and clinical results, we propose that the mechanism of action of *in situ* Ad-REIC is as follows: intratumoral injection of Ad-REIC induces massive apoptosis of cancer cells due to ER stress and provides an ideal presentation of possible cancer antigens to the host immune system. Secreted REIC protein at the tumor site creates an optimal environment, mediating tumor-associated, antigen-specific cytotoxic T cells. In addition, the overproduction of IL-7 by cancer-associated fibroblasts activates innate immunity involving NK cells (22, 33).

Since the induction of activated DCs and antigen-specific CTLs is crucial for the development of therapeutic cancer vaccines by Ad-REIC, we investigated this process carefully in a mouse tumor model using E.G7 expressing OVA. The expression of REIC/Dkk-3 is lost in E.G7 tumors. In vitro susceptibility of E.G7 to Ad-REIC was low and a high MOI (500 MOI) was needed to induce sufficient apoptosis in the in vitro study (Figure 1). Nevertheless, remarkable in vivo killing effects by Ad-REIC on subcutaneous E.G7 tumor were obtained (Figure 2).

13In general, naive CD8+ T cells interact with TAA-loaded APCs and acquire an effector 14function (34). In this murine model, cell-associated OVA might be internalized by DCs in the treatment process of Ad-REIC and OVA peptide with the major histocompatibility complex 1516(MHC) class I was presented to naïve CD8+ T cells. The resulting OVA-specific CTLs were 17detected successfully by a peptide-MHC tetramer assay, which is the most reliable assay for investigating antigen-specific CTL (Figure 3). In addition, CD8+ T cells, including 18 OVA-tetramer+ cells, showed IFN- γ production capacity, which is a functional surrogate marker 1920for identifying peptide-specific CTL activity.

As cross-presentation is a critical step for the priming of effective anti-cancer T cell responses, we performed an *in vitro* cross-presentation assay using OT-1 cells as indicated in our previous study (35, 36). Unexpectedly, OT-1 cells showed poor IFN- γ production after co-culture with DCs from Ad-REIC treated mice (data not shown). Nevertheless, DCs were CD8+ rDCs and CD103+ mDCs phenotype, whose expression levels of CD86 were higher than those of DCs from control Ad-LacZ treated mice (Figure 5). CD86 is an activated marker and both CD8+

1 rDCs and CD103+ mDCs are known to have cross-presentation ability (37). Therefore, it is 2 highly plausible that Ad-REIC gene therapy influences the cross-presentation pathway in DCs. 3 In our previous study, recombinant REIC/Dkk-3 protein with cytokine-like function was 4 shown to induce the differentiation of human CD14+monocytes into a novel cell type, DC-like 5 cells (12). The intratumoral administration of REIC/Dkk-3 protein significantly suppressed 6 subcutaneous RM9 tumor growth with CD11c+ and CD8+ (dendritic and killer T cell marker, $\overline{7}$ respectively) cell accumulation as determined by an immunohistochemical analysis (12). 8 Furthermore, a 17 kDa cysteine-rich core domain was recently shown to be sufficient for the 9 induction of DC-like cell differentiation from monocytes (13). Concomitant with the 10 differentiation of DCs, the REIC/Dkk-3 protein induced the phosphorylation of glycogen 11 synthase kinase 3β (GSK- 3β) and signal transducers and activators of transcription (STAT) 3 12and STAT5 at a level comparable to that of GM-CSF (13). According to these observations, it is 13possible that REIC/Dkk-3 protein secreted from tumors influences DC activation and differentiation. 14

Consequently, activated DCs in the TDLNs sufficiently induced OVA-specific CTLs, which 1516circulated throughout the body and infiltrated into non-injected tumor in the present dual 17tumor-bearing murine model (Figure 6). In this process, CD8+ CTLs play and essential roles in the anti-tumor effects based on the results with anti-CD8 antibody to deplete the T cell functions. 18 Using mouse prostate cancer model with human PSA-expressing tumor, we further confirmed 1920the generalizability of the current experiments in the other tumor type of prostate cancer. 21Challenges for the routine detection of antigen-specific CTLs induced by Ad-REIC have not 22been successful in our phase I/IIa clinical trial in prostate cancer. However, the present findings 23on OVA-specific CTLs induced by Ad-REIC in the E.G7 mouse model and on PSA-expressing 24tumor mouse model are extremely helpful to support our promising clinical results obtained and 25proposed action mechanism of in situ Ad-REIC to generate personalized therapeutic cancer 26vaccines.

1 • Materials and methods

2 Mice and cell lines

C57BL/6 female mice (ages: 6-8 weeks), were obtained from CLEA Japan. All mice were
maintained under specific pathogen-free conditions in Okayama University. E.G7 is an OVA
cDNA transfected derivative of the EL4 (methylchoranthlene-induced thymoma of C57BL/6
[H-2^b] origin) cell line. The E.G7 cells were kindly provided by Prof. Udono. The E.G7 cells
were maintained with RPMI-1640 containing 10% FCS, 2 mM l-glutamine, 1 mM sodium
pyruvate, 0.1 mM non-essential amino acid, penicillin-streptomycin, 2-mercaptoethanol and 400
µg/ml G418.

10

11 Adenovirus vector carrying REIC/Dkk-3

To induce the overexpression of REIC/Dkk-3, an adenovirus vector carrying the REIC/Dkk-3 gene (Ad-REIC) was created. A full-length cDNA was integrated into a cosmid vector pAxCAwt and transferred into an adenovirus vector using the COS-TPC method (Takara Bio, Shiga, Japan). An adenovirus vector carrying the LacZ gene (Ad-LacZ) was used as a control.

16

17 Apoptosis assay

E.G7 cells $(1.0 \times 10^5 \text{ cells})$ were seeded in 6-well plates and incubated in culture medium for 24 h. The cells were then treated with Ad-LacZ or Ad-REIC at the indicated MOI in 2 ml of medium. After 24 h of incubation, the apoptotic cells were visualized by Hoechst 33342 staining, and the apoptotic rate was analyzed as previously described (38, 39)

22

23 Western blot analysis

Total protein was extracted from the treated tumor tissue and Western blotting was performed as described previously (14). Proteins were identified with the use of the mouse monoclonal anti-human REIC/Dkk-3 antibody, which was raised in our laboratory, at 1000 x dilution. 1

2 In vivo experiments

3	E.G7 cells were suspended in serum-free medium and inoculated intradermaly into the left
4	back of mice (1 x 10^6 cells in 200 µl). One week after injection, when the tumor reached ~10
5	mm in diameter, Ad-REIC or Ad-LacZ was injected intratumorally at a dose of 1×10^9 pfu in
6	(40)100 µl of PBS. Mice received a 2nd injection of Ad-REIC or Ad-LacZ 2 days after the 1st
7	injection. The size of the tumors was measured every 2 days. The tumor volume was calculated
8	using an empirical formula, $V = 1/2 x$ (the shortest diameter) x (the shortest diameter) x (the
9	longest diameter). The experiments were perfomed according to the guidelines of Okayama
10	University. In the CD8-depleting experiments, mice were anesthetized with ether, after which
11	anti-Lyt-2.2 (CD8) antibody diluted in PBS to a total dose of 200 μ L per mouse was injected
12	intraperitoneally (24). In the other experiments, mouse prostate cancer cell line, PSA-RM9,
13	which is stably transfected and expresses human prostate specific antigen (PSA), was used as
14	previously described (40).

15

16 Flow cytometric analysis

17The following fluorochrome-labeled anti-mouse mAbs were used. PerCP/Cy5.5 conjugated 18anti-mouse CD19 (BioLegend), PE-Cy7 conjugated anti-mouse CD8a(BioLegend), APC-Cy7 19conjugated anti-mouse CD11c (BioLegend), FITC conjugated anti-mouse MHC Class II 20(I-A/I-E) (eBioscience), PE conjugated anti-mouse CD11b (BioLegend), APC conjugated 21anti-mouse CD103 (BioLegend) and PE conjugated anti-mouse CD86 (BioLegend) were used 22for detection of DC populations. Cells were washed and incubated with mAbs for 30min at 4 °C 23in 5 mM EDTA and PBS containing 2 % FCS (FACS buffer). All stained cells were acquired on 24a FACSCanto with the FACSDiva software program (BD Biosciences, Palo Alto, CA, USA) and 25analyzed using the FlowJo software program (TreeStar, San Carlos, CA).

1 Tetramer assays

 $\mathbf{2}$ For the analysis of OVA-specific CTL frequency, lymphocytes were obtained from the spleen 3 and TDLNs and TILs on day 2 after the last treatment. The TIL and TDLN cells were washed twice in FACS buffer and incubated with PE-conjugated H-2K^b OVA tetramer (MBL) for 30 4 min at RT. After tetramer staining, PE-Cy7 conjugated anti-mouse CD4 (BioLegend) and 5 6 APC-Cy7 conjugated anti-mouse CD8 α (BioLegend) were used for cell surface staining. After 7 harvesting the splenocytes from the mice on the indicated days, the cells were co-incubated with 8 OVA257–264 peptide (SIINFEKL) for 5 days. The incubated cells were also stained by OVA 9 tetramer and cell surface Ab. The frequency of OVA-specific CD8+ cells were analyzed on a 10 FACSCanto II system.

11

12 Intracellular cytokine assay

For the DLN and naive T cell intracellular cytokine staining, samples were restimulated with 50 ng/ml PMA and 500 ng/ml ionomycin (Sigma-Aldrich) for 4 h. The cells were surface stained with APC-Cy7 conjugated anti-mouse CD8 α (BioLegend). The cells were washed, fixedand permeabilized with Cytofix/Cytoperm buffer and intracellular staining was performed with PE conjugated anti-mouse IFN- γ (eBioscience). Finally, The cells were analyzed with a FACSCanto II system.

19

20 Statistical analysis

All data expressed as the means \pm s.e.m. and representative of at least two different experiments. The statistical differences between the groups were assessed by analyzing means of replicates using the two-tailed Student's t test. A P value of <0.05 indicated that the value of the test sample was significantly different from that of the relevant controls.

25

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 3 26462413.
- 4
- 5 Conflict of Interest
- 6 Okayama University and Momotaro-Gene Inc. are applying for patents on the Ad-REIC systems.
- 7 Drs. M. Watanabe, Y. Nasu and H. Kumon are the inventors of the patents and own stock in
- 8 Momotaro-Gene Inc.

	1	•	Figure	legends
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2	Figure 1. The induction of apoptosis in E.G7 tumor cells after infection with Ad-REIC
3	(a) Hoechst staining of Ad-REIC or Ad-LacZ treated E.G7 cells. E.G7 cells were infected with
4	Ad-LacZ or Ad-REIC at 100 MOI and 500 MOI. Apoptotic cell death was examined in terms of
5	changes in cell morphology which were observed by Hoechst 33342 staining.
6	(b) The quantitation of the apoptotic cells is shown. Vertical bars, standard deviation. The P
7	value was calculated using the paired t-test.
8	
9	Figure 2. The antitumor effect of in situ Ad-REIC gene therapy
10	(a) The experimental schedule of the Ad-REIC treatment is shown. After the tumor volume
11	reached 5mm, 1 x 10 ⁹ pfu of Ad-REIC or Ad-LacZ was intoratumorally injected two times.
12	(b) A Western blot analysis of the expression of REIC/Dkk-3 protein in E.G7 tumors after
13	injection. Tubulin was used as a loading control.
14	(c) The growth curve of the E.G7 tumors. The tumor size was monitored 2 weeks after Ad-REIC
15	or Ad-LacZ treatment into E.G7 bearing mice. The tumor volume was measured as described in
16	the Materials and Methods section. A significant difference was observed between the results of
17	Ad-REIC and Ad-LacZ treatment (*P<0.05).
18	
19	Figure 3. The detection of OVA-specific CTL induction using by OVA tetramer assay
20	Tumor and spleen were harvested and immediately digested. Single cell suspensions were
21	stained with OVA tetramer.
22	(a) TILs were collected 2 days after treatment. After gating on CD8 lymphocytes, OVA-specific
23	T cells were analyzed by flow cytometry.
24	(b) Spleen cells were stimulated with OVA peptide for one week.
25	(c) The proportion of OVA-specific CTLs in the tumor and spleen are shown. The P value was
26	calculated using the paired <i>t</i> -test.

1

2 Figure 4. The effector function of CTLs

a) TILs and TDLNs were isolated from the treated mice on day 2 after the injection of
Ad-REIC or Ad-LacZ injection. For intracellular staining, all cells were restimulated with
PMA/ionomycin for 4 h before analysis. After stimulation, all samples were stained for
anti-CD8 and intracellular IFN-γ.

- 7 (b) The mean cell populations of CD8+ and IFN-γ producing cells in response to 4h of
 8 stimulation with PMA/Ionomycin.
- 9 (c) The proportion of IFN-γ producing cells in TILs (left) and TDLNs (right).
- 10 * Student's unpaired *t*-test, P < 0.05, averaged from three separate experiments.

11

- 12 Figure 5. The phenotype and frequency of DC populations in TDLNs
- 13 (a) A flow cytometric analysis of DCs purified in TDLN of Ad-REIC or Ad-LacZ mice, and
- 14 stained with mAbs for CD11c, MHC class II and CD86.
- 15 (b) Percentage of each population among CD19- CD11c^{int} MHCII+ cells and among CD19-
- 16 $CD11c^{high}$ MHCII+ cells. The mean \pm SD in three mice is shown.
- 17 (c) Histograms showing CD86 expression in migratory DCs (left) and resident DCs (right).

18

19 Figure 6. The antitumor effects in the Ad-REIC non-injection side

20 (a) The experimental schedule of the Ad-REIC treatment is shown. E.G7 cells were inoculated

- 21 into the left and right back. 1.0 x 10⁹ pfu of Ad-REIC or Ad-LacZ were intoratumorally injected
- in only right side tumor.
- 23 (b) Non-injected TILs were isolated on post-treatment day 2. All cells were stained with
- anti-CD8 and OVA tetramer beads and were analyzed by flow cytometry. The overall frequency
- 25 of the OVA tetramer+ T cells in the non-injection side tumor. The P value was calculated using

(c) The tumor growth curves and a macroscopic view of E.G7 tumor. The mean volume of the
 tumors was calculated and the tumor growth curves are shown. *A significant difference was
 observed between Ad-REIC group and the control vector groups.

(d) The tumor growth curves of E.G7 tumor. The mean volume of the tumors was calculated and
the tumor growth curves are shown. *A significant difference was observed in the directly
treated tumor between Ad-REIC group and Ad-REIC plus anti-CD8 antibody added groups. [†]A
significant difference was observed in the opposite (non-injected) tumor between Ad-REIC
group and Ad-REIC plus anti-CD8 antibody added groups.

(e) The tumor growth curves of PSA-RM9 tumor. The mean volume of the tumors was
calculated and the tumor growth curves are shown. *A significant difference was observed in
the directly treated tumor between Ad-REIC group and the control vector groups. [†]A significant
difference was observed in the opposite (non-injected) tumor between Ad-REIC group and the
control vector groups.

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Figure 1.

The induction of apoptosis in E.G7 tumor cells after infection with Ad-REIC

(a)



LacZ





Figure 2.

The antitumor effect of *in situ* Ad-REIC gene therapy



(c)



Figure 3.

The detection of OVA-specific CTL induction using by OVA tetramer assay



Figure 4. The effector function of CTLs



Figure 5. The phenotype and frequency of DC populations in TDLNs

(a)



CD86



rDC

Figure 6. The antitumor effects in the Ad-REIC non-injection side



Figure 6. The antitumor effects in the Ad-REIC non-injection side



 $^{+}$ p < 0.05 compared between REIC non-injection and α CD8 + REIC non-injection side



* p < 0.05 compared between Ad-REIC injection and Ad-LacZ injection

 † p < 0.05 compared between Ad-REIC non-injection and Ad-LacZ non-injection side