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ORIGINAL ARTICLE

Adenovirus-mediated REIC/Dkk-3 gene transfer inhibits tumor growth and metastasis in an orthotopic prostate cancer model

K Edamura[1], Y Nasu[1], M Takaishi[2], T Kobayashi[1], F Abarzua[1], M Sakaguchi[2], Y Kashiwakura[3], S Ebara[1], T Saika[1], M Watanabe[1], N-H Huh[2] and H Kumon[1]

[1]*Department of Urology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan;* [2]*Department of Cell Biology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan and* [3]*Center for Gene and Cell Therapy, Okayama University Hospital, Okayama, Japan*

We had previously reported that REIC/Dkk-3, a member of the Dickkopf (Dkk) gene family, works as a tumor suppressor. In this study, we evaluated the therapeutic effects of an intratumoral injection with adenoviral vector encoding REIC/Dkk-3 gene (Ad-REIC) using an orthotopic mouse prostate cancer model of RM-9 cells. We also investigated the *in vivo* anti-metastatic effect and *in vitro* anti-invasion effect of Ad-REIC gene delivery. We demonstrated that the Ad-REIC treatment inhibited prostate cancer growth and lymph node metastasis, and prolonged mice survival in the model. These therapeutic responses were consistent with the intratumoral apoptosis induction and *in vitro* suppression of cell invasion/migration with reduced matrix metalloprotease-2 activity. We thus concluded that *in situ* Ad-REIC/Dkk-3 gene transfer may be a promising therapeutic intervention modality for the treatment of prostate cancer.

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Keywords: Dkk-3; REIC; prostate cancer; apoptosis; metastasis; invasion

Introduction

Prostate cancer is the most common cancer and the second leading cause of cancer-related deaths in the male population of the United States.[1] Cancer metastasis is a major clinical issue, as it accelerates death in cancer patients and is accompanied with troublesome and often painful clinical symptoms. In spite of androgen deprivation therapy, radiation and cytotoxic chemotherapy, disease progression and metastasis occur in most cases. Therefore, novel and effective therapies against prostate cancer are urgently needed.

The malignant progression of prostate cancer is often accompanied by the downregulation of apoptosis[2] and increased metastatic activity, such as invasion and motility.[3] Cancer cells modulate the apoptotic and metastatic processes by producing both positive and negative effectors,[4,5] and a number of proteins exhibit potent anticancer effects involved in these processes.[6–9] REIC/Dkk-3, a member of the Dickkopf (Dkk) gene family known to interfere with Wnt signaling via Wnt receptors,[10,11] was recently reported to play a distinct role in apoptosis induction[12,13] and invasion/motility inhibition.[14,15] Its expression was initially found to be downregulated in human cancer cells in comparison to the levels in normal cells,[16–19] and similar findings were documented when analyzing normal prostate and cancer materials.[13,20] Moreover, REIC/Dkk-3 expression in prostate cancer consistently shifts to be downregulated at the critical transition from non-invasive disease to highly invasive disease.[13,20] These findings strongly suggest that REIC/Dkk-3 is a tumor-suppressor gene and an attractive therapeutic protein to inhibit prostate cancer progression.

Gene therapy has been used in clinical trials for human diseases and it is considered to have a novel and attractive therapeutic potential. Our group has demonstrated the feasibility of using adenoviral and adeno-associated viral vectors to deliver therapeutic genes to animals and humans.[21–24] Specifically regarding gene therapy for the treatment of prostate cancer, adenovirus-mediated gene delivery has been well reported and indicates a definite advantage in terms of a high transduction efficacy.[13,25] We herein generated adenoviral vectors encoding both mouse and human REIC genes and attempted to suppress the mouse prostate cancer growth in the orthotopic mice model by intratumoral REIC gene transfer. We investigated further the anti-metastatic effect of *in vivo* Ad-REIC gene therapy to demonstrate its therapeutic utility against prostate cancer progression.

Correspondence: Dr Y Nasu, Department of Urology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8558, Japan. E-mail: ynasu@md.okayama-u.ac.jp
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Materials and methods

Animals

C57BL/6 adult male mice were purchased from SLC Inc. (Hamamatsu, Japan) and maintained in a specific pathogen-free environment with free access to food and water at the laboratory animal center of Okayama University. They were allowed to adapt to their environment for more than 1 week beginning the experiments were begun. The animals were housed and handled in accordance with the Okayama University Animal Research Committee Guidelines.

Cell line

RM-9 mouse prostate cancer cell line was kindly provided by Dr TC Thompson (Baylor College of Medicine, Houston, TX). The cell line was derived from a primary prostate tumor induced in the Zipras/myc-9 infected mouse prostate reconstitution model system using C57BL/6 mice.[26] The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) with 10% fetal bovine serum (FBS; Gibco-BRL, Grand Island, NY), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, without the use of antibiotics.

Adenovirus vector carrying REIC/Dkk-3

For the overexpression of mouse or human REIC/Dkk-3, a full-length cDNA was integrated into a cosmid vector pAxCAwt and transferred into an adenovirus vector by the COS-TPC method (Takara Bio, Shiga, Japan). An adenovirus vector carrying LacZ gene was used for the control, as described previously.[13]

In vivo *experiments*

For the orthotopic tumor formation, C57/BL6 mice were intraperitoneally anesthetized with sodium pentobarbital (0.1 mg/g body weight) and placed in the supine position. A low abdominal transverse incision was made and the bilateral dorsal lobes of prostate were exposed. Following the trypsinization of RM-9 cells, 5.0×10^3 cells in 10 μl of Hanks' balanced salt solution were injected using a 30-guage needle and a 25-μl glass syringe (Hamilton Co., Reno, NV) directly into the right dorsal lobe of the prostate. A clearly recognizable bleb within the injected prostatic lobe was considered as a sign of a technically satisfactory injection. The abdominal wound was closed with stainless steel clips (Autoclip; Becton Dickinson Co., Sparks, MD). One week after the RM-9 cell injection, when the tumor diameter reached 5 mm, 1.2×10^8 PFU of an adenovirus vector carrying full-length mouse or human REIC/Dkk-3 cDNA and LacZ (Ad-mREIC, Ad-hREIC and Ad-LacZ) was intratumorally injected in a 25 μl vehicle. The same volume of phosphate-buffered saline (PBS) was injected as a negative control.

Measurement of the orthotopic tumor growth

The size of the tumors was measured every 3 days after the treatment using the transrectal ultrasonography (TRUS) that we previously established.[27] A 20-MHz-based radial scan probe integrated into a 6 Fr. catheter (ALOKA Co. Ltd, Tokyo, Japan) and an ultrasound unit (SSD-550; ALOKA Co. Ltd) were used for this study. The 20-MHz transducer provides high-resolution images with an axial resolution of 0.2 mm and revolves at 15 frames per second, producing a 360° cross-sectional real-time image. The tip of the catheter was lubricated with ultrasound gel and then inserted into the rectum while the mouse was secured without anesthesia. The tumor volumes were calculated using the formula $1/2 \times$ (the shortest diameter)$^2 \times$ (the longest diameter).

Histological analysis and apoptosis assay

For the analysis of lymph node metastasis, the mice were killed and retroperitoneal lymph nodes were removed, fixed by formalin and embedded for paraffin sections. The sections (5 μm) were stained with hematoxylin and eosin, and examined histologically. To identify the cells undergoing apoptosis in orthotopic RM-9 tumors, an *in situ* TdT-mediated dUTP nick end labeling (TUNEL) assay was performed using a kit (Roche, Indianapolis, IN) as described previously.[13] Briefly, the tumor tissue was cut, placed in the optical cutting temperature (OCT) compound and snap-frozen in liquid nitrogen. The frozen sections (10 μm) were then fixed with methanol for 30 min at room temperature (RT), washed and permeabilized with PBS containing 0.1% Triton X-100 and stained with the TUNEL reaction mixture.

Invasion assay

Invasion assay with a Matrigel-coated membrane was performed using a 24-well invasion chamber system (BD Biosciences, Bedford, MA), according to the manufacturer's instructions. The cells were trypsinized and seeded in the upper chamber at 2.5×10^5 cells/well in serum-free DMEM. The vector carrying LacZ or REIC/Dkk-3, at a multiplicity of infection (MOI) of 10, was added immediately after cell plating. DMEM supplemented with 50% FBS (used as a chemoattractant) was placed in the bottom well. Incubation was carried out for 24 h at 37°C in humidified air with a 5% CO_2 atmosphere. The cells were allowed to migrate through a porous, Matrigel-coated membrane (BD Biosciences). After the incubation, the chamber was removed, and invading cells on the bottom side of the membrane were fixed with methanol at RT and stained with hematoxylin. The number of invading cells was determined by counting five high-power fields ($\times$ 200) on each membrane and calculated as the mean number of cells per field. The invasion index, corrected for the cell motility, was calculated using the following formula:

$$\frac{\text{No. of cells invading through a Matrigel} - \text{coated membrane} \times 100}{\text{No. of cells migrating through an uncoated (control) membrane}}$$

Motility assay

The cell motility assay was performed using a porous, uncoated membrane (BD Biosciences) instead of the Matrigel-coated membrane used in the invasion assay. A total of 1×10^5 cells/well were placed in the upper

chamber in serum-free DMEM. The vector at a 10 MOI was added immediately after cell plating. DMEM supplemented with 50% FBS was placed in the bottom well. The cells were allowed to migrate through a porous, uncoated membrane (BD Biosciences) for 24 h at 37°C. The number of migrating cells was determined by counting five high-power fields (× 200) on each membrane and then calculated as the mean number of cells per field.

Zymography

The gelatinolytic activity of matrix metalloproteases (MMPs) in the conditioned medium of RM-9 cell line was determined using zymography. The subconfluent cells were treated or not treated with Ad-REIC or Ad-LacZ at 10 MOI in DMEM supplemented with 10% FBS. After cultivating the cells for 24 h, to remove any excess amount of serum proteins in the culture medium, the cells were washed two times with Opti-MEM (Gibco/BRL, Carlsbad, CA) and thereafter maintained in the same medium for 24 h. Next, 10 ml of the conditioned medium in each was collected, concentrated to 100 μl by centrifugation method with a Centricon (Amicon Ultra-15 Ultracell-10k; Millipore, Billerica, MA) and mixed with 50 μl of 3 × sodium dodecyl sulfate (SDS) sample buffer. Subsequently, the 10 μl mixtures were applied to 7.5% SDS-polyacrylamide slab gel containing 1 mg/ml gelatin (Sigma Chemical Co., St Louis, MO). The gel was run at RT using a vertical electrophoresis system for separation by the molecular mass. After electrophoresis, the gel was soaked in 2.5% Triton X-100 for 1 h to remove SDS and incubated in 50 mmol/l Tris–HCl (pH 8.0) containing 0.5 mmol/l $CaCl_2$ and 1 μmol/l $ZnCl_2$ for 16 h at 37°C. The gel was then stained with 1% Coomassie brilliant blue G250 (CBB-G250). After the staining, the gel was further treated with 10% methanol and 5% acetic acid to destain. The proteolytic activity was detected as bands on a blue background of the CBB-stained gel. As a loading control, similarly applied SDS-polyacrylamide gel was run, electroblotted onto a nitrocellulose membrane, as described previously,[13] and stained with CBB-G250.

Statistical analysis

The data are shown as the mean ± s.e.m. Mann–Whitney *U*-test or Fisher's exact test was performed for the statistical analysis between the two groups. Kaplan–Meier curves and log-rank tests were used for the survival analysis. Differences were considered significant if $P<0.05$.

Results

Intratumoral injection of Ad-REIC inhibits prostate tumor growth and induces apoptosis

We recently revealed that Ad-hREIC-mediated overexpression of REIC protein in cancer cells could efficiently induce apoptosis *in vitro* and in the subcutaneous prostate tumor model.[13] Therefore, the current study, using an orthotopic prostate cancer model, was performed as a preclinical study of this gene therapy. We first evaluated whether or not Ad-REIC treatment could inhibit RM-9 tumor growth in the model. As the prostate cancer size in clinical patients is routinely determined by TRUS, we also used this same system throughout this study. The high-resolution TRUS allowed for simple and reliable monitoring of the *in situ* tumor size (Figure 1a).

When the orthotopic tumors were formed, the mice were divided into four treatment groups with comparable tumor ranges. In the Ad-mREIC- or Ad-hREIC-treated group, tumor growth was significantly suppressed in comparison to the control groups (Figure 1b). The growth curve of the Ad-LacZ-injected tumors was similar to that of PBS-treated tumors (Figure 1b), and all of the mice in these groups lived until day 17 after the treatment. In the next experiment, the tumors were harvested and examined by TUNEL staining. Only a few apoptotic cells were observed in the tumors injected with PBS or with Ad-LacZ on day 3, whereas many cells were positive in the

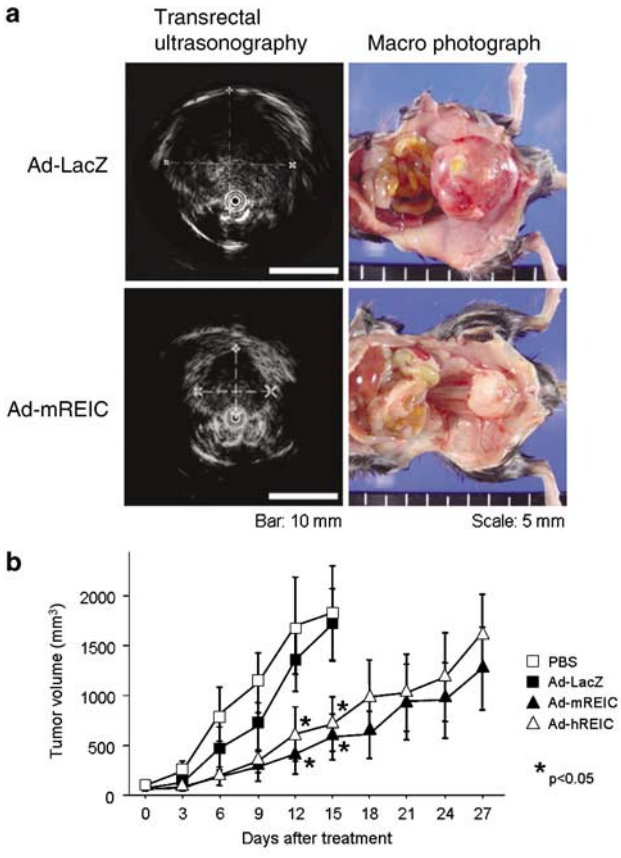

Figure 1 Suppression of tumor growth by intratumoral Ad-REIC delivery (**a** and **b**). (**a**) Representative macroscopic and transrectal ultrasonographic views of orthotopic RM-9 tumors on day 15 after Ad-mREIC or Ad-LacZ treatment. (**b**) Orthotopic RM-9 tumors were formed and injected intratumorally with Ad-mREIC, Ad-hREIC, Ad-LacZ or PBS on treatment day 0. Tumor size was measured by TRUS; data represent the average of five individual mice in each group; bars, ± s.e.m. A significant difference was observed (*$P<0.05$) between the Ad-REIC and Ad-LacZ treatments.

Ad-mREIC-injected tumors (Figure 2a). A quantitative analysis of apoptosis was performed in the tumor sections of the Ad-mREIC-, Ad-LacZ- or PBS-treated groups at the indicated times. As the number of apoptotic cells correlated with the number of $\times 200$ power fields in which they were found, the data were expressed as the number of positive cells/$\times 200$ power fields. On day 3, an approximately sevenfold increase in apoptotic activities was detected in the Ad-mREIC-treated tumors in comparison to the control groups (Figure 2b).

In situ *REIC gene transfer suppresses spontaneous lymph node metastases*

We previously disclosed that RM-9 cell implantation in the mouse prostate led not only to the formation of a primary tumor, but also to spontaneous metastases to the para-aortic lymph nodes.[22] At the time of killing of the current orthotopic model mice, we incidentally discovered a smaller incidence of para-aortic lymph node swelling in the animals treated with intratumoral Ad-REIC injection, in comparison to the controls. We therefore noticed the possibility that an Ad-REIC treatment may inhibit lymph node metastases of the RM-9 cells as well as the tumor growth, and then attempted to test this hypothesis. As it seems that mice with larger orthotopic prostate tumors show more incidence of lymph node metastasis, we considered that a killing at the same tumor volume would be necessary to compare the metastatic status between the treatment groups. When the tumor volume reached an average of 750 mm^3 by TRUS measurement, the mice in each group were killed and retroperitoneal lymph nodes were histologically examined (Figure 3a). Thereafter, the number of metastatic lymph nodes and the incidence of metastasis were analyzed to determine the *in vivo* anti-metastatic effect of Ad-REIC treatment. The number of lymph nodes positive for metastasis was 3.2 ± 0.6 in the Ad-LacZ group, and it significantly decreased to 0.9 ± 0.4 in the Ad-mREIC group (Figure 3b). In addition, only 42–45% of mice in the Ad-REIC-treated groups showed histologically proven nodal metastasis, while metastasis was seen in 82% of the control animals (Figure 3b).

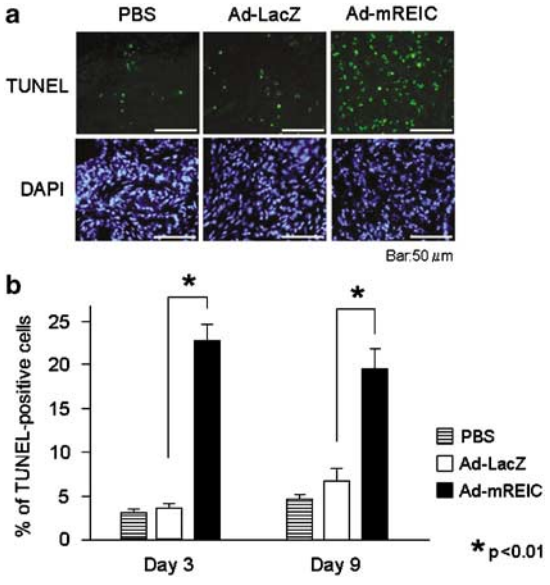

Figure 2 Increased apoptotic rates in Ad-mREIC-treated tumors (**a** and **b**). (**a**) Representative TUNEL staining from the Ad-mREIC-, Ad-LacZ- or PBS-treated RM-9 tumors on day 3 is shown. The sections were counterstained with DAPI. (**b**) Quantitative analyses of TUNEL-positive cells were carried out using the data from TUNEL staining of RM-9 tumors on days 3 and 9 after the treatments. The data represent the average of five individual mice in each group; bars, $\pm$s.e.m. There was a significant difference (*$P<0.01$) between the Ad-mREIC and Ad-LacZ treatments. PBS, phosphate-buffered saline.

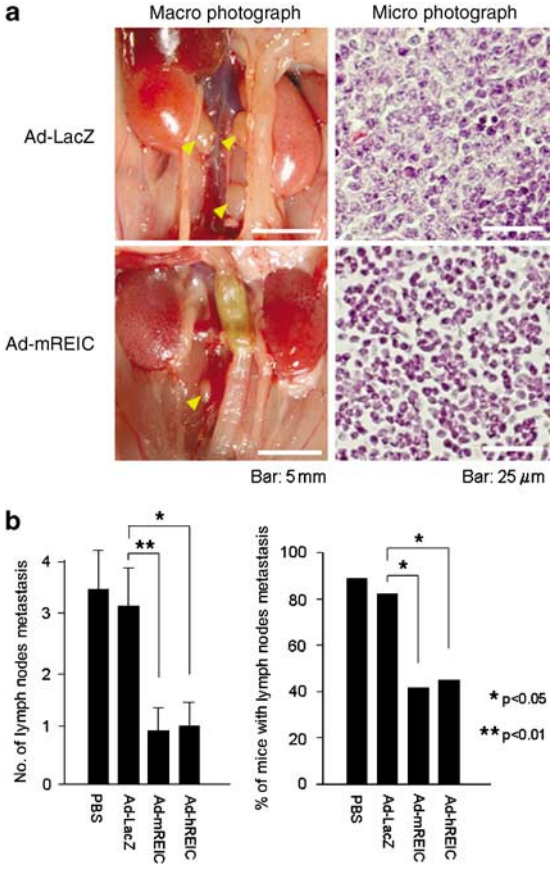

Figure 3 Suppression of cancer metastasis by intratumoral Ad-REIC delivery (**a** and **b**). (**a**) Representative macroscopic and microscopic views of RM-9 metastasis in the retroperitoneal lymph nodes after Ad-mREIC or Ad-LacZ treatment. The mice in each group were killed when the tumor volume reached an average of 750 mm^3 by TRUS measurement. Arrowheads indicate the retroperitoneal lymph nodes. The lymph node sections were stained with hematoxylin and eosin. Histological RM-9 metastasis was revealed in the lymph node of the Ad-LacZ-treated mouse but not of the Ad-mREIC-treated mouse. (**b**) The number of metastatic lymph nodes and incidence of metastasis were analyzed when the tumor volume reached an average of 750 mm^3 by TRUS measurement. The data represent the average of 10–12 individual mice in each group; bars, $\pm$s.e.m, or percentage of animals with lymph node metastasis. PBS, phosphate-buffered saline; TUNEL, TdT-mediated dUTP nick end labeling.

REIC gene transfer inhibits the invasion and migration of RM-9 cells in vitro

On the basis of the current results that *in situ* REIC gene delivery significantly suppressed the spontaneous lymph node metastases *in vivo* (Figure 3b), we next examined the *in vitro* effects of Ad-REIC treatment on RM-9 cell invasiveness and motility, which are major factors that determine the metastatic activity.[28] The Matrigel invasion assay system has been used to analyze the three-step cell invasion activity: (a) adhesion to a substrate; (b) dissolution of the extracellular matrix (ECM); and (c) migration.[14,29] Using this assay, we observed a significant decrease in the invasion activity of Ad-mREIC- or Ad-hREIC-treated RM-9 cells, in comparison to the control cells (Figure 4a). Because the Ad-REIC treatment suppressed RM-9 cell invasion, we next examined its inhibitory effect on cell motility, one of the components of the cancer invasion process, by transwell migration assay. RM-9 cells placed in the upper chamber were induced to migrate across 8 μm membrane pores in response to the chemoattractant. As expected, the number of motile RM-9 cells was significantly decreased after the Ad-REIC treatment (Figure 4a). Neither distinctive cellular morphological changes nor any significant apoptotic induction confirmed by TUNEL assay was detected under the experimental conditions.

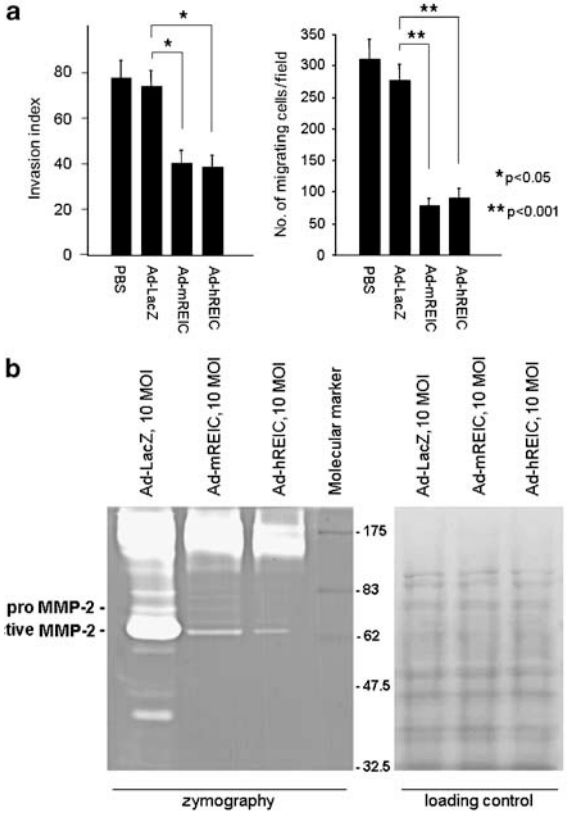

Figure 4 Decreased invasion and migration activity of RM-9 cells after Ad-REIC treatment. (**a**) *In vitro* invasion assay through Matrigel and transwell migration assay were performed as described in Materials and methods section. Data represent the average of three independent experiments in each group; bars, $\pm$s.e.m. (**b**) Extracellular secretion of both the pro and active forms of MMP-2 was determined by zymography. As a loading control, a similarly loaded gel was run, electroblotted onto a nitrocellulose membrane and stained with CBB-G250. The experiment was carried out in triplicate.

Ad-REIC treatment suppresses MMP-2 secretion in RM-9 cells

It is well known that both invasion and metastasis of cancer cells occur through the activation of MMPs, such as MMP-2 and -9, which degrade the ECM to overcome the ECM barrier at cell migration.[5] We preliminarily examined MMP-2 expression in the Ad-REIC-treated cells by western blot analysis and disclosed the reduced expression in comparison to that of the control cells (data not shown). To determine whether or not the inhibition of RM-9 invasion by Ad-REIC relies on a downregulated proteolysis by the MMPs, we assessed MMPs activities by gelatin zymography. In the control RM-9 cells treated with Ad-LacZ, zymographic bands consistent with pro and active forms of MMP-2 were observed at the expected molecular weight[30] (Figure 4b). In the Ad-REIC-treated cells, the MMP-2 band density of both forms was evidently decreased (Figure 4b). In particular, the level of MMP-2 active form at 64 kDa showed a significant decline after the Ad-mREIC or Ad-hREIC treatment.

In situ *REIC gene therapy prolongs the survival of mice in an orthotopic prostate cancer model*

To further confirm the therapeutic utility of Ad-REIC gene therapy, we analyzed the survival of the treatment groups. The tumor-bearing mice treated with Ad-mREIC or Ad-hREIC had a significantly prolonged survival in comparison to the control (Figure 5). Therefore, both the *in vivo* ablation of prostate tumor volume and inhibition of lymph node metastasis were achieved using an adenovirus-mediated gene delivery of REIC with a consequently improved survival.

Discussion

We previously cloned the REIC/Dkk-3 gene, whose expression was shown to be markedly downregulated in

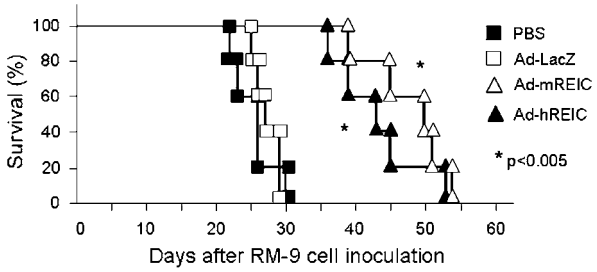

Figure 5 Long-term survival of RM-9 tumor-bearing mice after intratumoral Ad-REIC delivery. Kaplan–Meier curve is shown in the Ad-mREIC-, Ad-hREIC-, Ad-LacZ- and PBS-treated groups, with each group consisting of five mice. There was a significant difference ($P<0.005$) between Ad-REIC and Ad-LacZ treatments.

many human cancer cell lines.[16–19] By *in vitro* transfection experiments, it was also determined that REIC/Dkk-3 possessed not only antiproliferative activities[17,20] but also apoptotic effects against tumor cells.[12,13] Therefore, the growing evidence showing that REIC/Dkk-3 definitely works as a tumor suppressor prompted us to evaluate the possibility of *in situ* REIC/Dkk-3 gene therapy. As the RM-9 cells lack the endogenous REIC protein, confirmed by Western blot analysis (data not shown), and are suitable to establish an orthotopic tumor followed by spontaneous metastases, we used the cell line for this study. We herein demonstrated that the intratumoral Ad-REIC injection suppressed the prostate cancer progression with regard to tumor growth and the metastasis in the orthotopic prostate cancer model. In addition, using TRUS in the model, we could sufficiently monitor tumor growth and its suppression by REIC/Dkk-3 gene therapy.

As for the apoptotic function of REIC, we recently revealed the forced expression of REIC/Dkk-3 induced apoptosis in human prostate cancer cell lines lacking endogenous REIC/Dkk-3 expression but not in REIC/Dkk-3 proficient normal prostate epithelial and stromal cells.[13] The apoptosis involved c-Jun-NH_2-kinase (JNK) activation, mitochondrial translocation of Bax, reduction of Bcl-2 and the release of cytochrome *c* into the cytoplasm.[13] The overexpression of REIC/Dkk-3 induced apoptotic cell death in several types of human cancer cell through the activation of caspase-3, which is known to be a major apoptosis executor in the downstreaming of cytochrome *c*.[12] In this study, the TUNEL analysis disclosed a significantly increased apoptotic incidence in the Ad-REIC-treated RM-9 tumors in comparison to the control tumors. The high level of apoptotic cell population observed on day 3 after Ad-mREIC treatment was maintained on day 9, when the treated tumors showed definite reductions in size in comparison to the controls. Since we confirmed the mREIC protein expression in the day 3 tumor by western blotting (data not shown), these results coincide with previous reports in which a forced REIC expression induced cancer cell apoptosis in both *in vitro* and *in vivo* conditions.[12,13] Therefore, an important finding of the current study is that adenovirus-mediated REIC/Dkk-3 gene delivery induces apoptosis in the *in vivo* prostate cancer model, suggesting that the induction of apoptosis accounts for the growth inhibition and prolonged survival in Ad-REIC-treated mice.

We herein used an orthotopic syngeneic prostate cancer model together with mouse TRUS and could efficiently demonstrate the utility of intratumoral Ad-REIC treatment to prevent lymph node metastasis. According to our present knowledge, this is the first study to disclose the *in vivo* anti-metastatic activity of REIC/Dkk-3. It is conceivable that the suppression of cancer metastasis by *in situ* Ad-REIC injection contributed to the improved survival of the groups. To elucidate the anti-metastatic mechanisms, we performed *in vitro* invasion and migration assays and revealed the downregulated invasiveness and motility of Ad-REIC-treated RM-9 cells. These results are consistent with the reported studies in which REIC/Dkk-3 stable transfected cell lines of osteosarcoma and melanoma showed an inhibition of both the invasion and motile activity *in vitro*.[14,15]

MMP-2 is thought to be a key enzyme for degrading type IV collagen in the ECM and basement membrane that facilitates local invasion, metastasis of cancer cells and angiogenesis within the various types of tumors, including prostate cancer.[5,31,32] Zymography is often used to measure MMP-2 level and activity of the conditioned medium, and the result indicates a positive correlation with the cell invasion capacity.[33,34] We found that the Ad-REIC treatment inhibited the level of extracellular MMP-2 in RM-9 cells when determined by zymography, indicating that the suppressed *in vitro* invasion and *in vivo* metastasis are attributed to the downregulated MMP-2 activity, at least in part. As both the pro and active forms of MMP-2 were suppressed, it is conceivable that the Ad-REIC treatment could inhibit both MMP synthesis and activity. The question, however, remains as to how Ad-REIC downregulated the MMP-2 level and/or activity in the RM-9 prostate cancer cells. MMPs synthesis/activation and tumor cell invasion have been associated with the activation of their regulators, such as the extracellular signal-regulated protein kinase (ERK)/mitogen-activated protein kinase and JNK/stress-activated protein kinase.[35,36] It is possible that these regulators are intracellularly activated either by direct interaction with REIC/Dkk-3 or indirectly through some, as yet, unknown target proteins of REIC/Dkk-3. On the other hand, it is interesting that human Dkk-1, which is a member of the Dkk gene family and has 39% homology to human REIC/Dkk-3,[10] also possesses anti-invasion activities.[37,38] Based on the well-investigated Dkk-1 function that the protein is a secreted antagonist of the Wnt cell signaling molecules and blocks the canonical Wnt–β-catenin signaling pathway,[10,11,39,40] we could not rule out the potential that the humoral REIC/Dkk-3 protein secreted after Ad-REIC treatment may block Wnt signaling, similar to the Dkk-1 protein. An important future step will be to clarify the mechanistic details of the Ad-REIC-triggered anti-invasion processes.

We herein demonstrated that the adenovirus-mediated REIC/Dkk-3 gene transfer suppressed prostate cancer progression in the orthotopic prostate cancer model. The results not only support our understanding of REIC/Dkk-3 as a tumor-suppressor gene and an attractive interventional target in this malignancy but also indicate that REIC/Dkk-3 deserves further exploration as a potential therapeutic tool in the other diseases of human cancer. Further experiments should examine the possibility of clinical Ad-REIC gene therapy in terms of its safety and toxicity.

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