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An N-terminal 78 amino acid truncation of REIC/Dkk-3 effectively induces apoptosis

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ABSTRACT

Overexpression of REIC/Dkk-3 (a tumor suppressor gene) induces cancer cell apoptosis through endoplasmic reticulum (ER) stress. Therefore, the identification of the portion of REIC/Dkk-3 that causes ER stress may be essential for the development of cancer treatment based on REIC/Dkk-3. Here, we made several truncated forms of REIC/Dkk-3 and investigated their therapeutic potentials against prostate cancer. Among three truncated forms, a variant comprising the N-terminal 78 amino acid region of REIC/Dkk-3 ($^{1-78}$REIC/Dkk-3) most strongly induced ER stress and apoptosis in human prostate cancer cells (PC3). For *in vivo* gene expression, we coupled a biodegradable polymer with naked DNA, which attained robust trans-gene expression in PC3-derived subcutaneous tumor. In therapeutic experiments, we demonstrated that multiple direct injections of polymer-conjugated $^{1-78}$REIC/Dkk-3 plasmid provoke ER stress and significantly reduced the subcutaneous tumor volume compared with the control group. We suggest this non-viral strategy may be an effective alternative to viral gene therapy.

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The Dickkopf (Dkk) gene family is suggested to prevent Wnt signaling, leading to suppression of pleiotropic effects in critical biological contexts including development, cell growth/differentiation, and cancer [1,2]. Although the four genes of the Dkk family have not been fully characterized, they are also likely to play important roles. We previously identified and characterized REIC/Dkk-3 as a tumor suppressor gene whose expression is reduced in many human cancers and demonstrated its anti-tumor function [3,4]. Adenovirus-mediated expression of REIC/Dkk-3 induced apoptosis through activation of c-Jun-NH_2-kinase (JNK) in cancer cells, but not in normal cells [5]. In contrast, naked DNA transfection of REIC/Dkk-3 can inhibit cell proliferation, but affects apoptosis only slightly [4]. Recently, we demonstrated that REIC/Dkk-3-induced JNK activation was largely mediated through endoplasmic reticulum (ER) stress in malignant mesothelioma [6]. Thus, augmentation of ER stress has the potential to be used as the basis of a non-viral therapeutic strategy involving REIC/Dkk-3 gene therapy.

Truncation mutations can cause unexpected changes in the molecular properties of a protein. For example, the N-terminal 95 amino acid region of insulin growth factor binding protein 3 ($^{1-95}$IGFBP-3) is a more potent apoptotic inducer than the full length protein [7]. Although the precise mechanism of $^{1-95}$IGFBP-3-induced apoptosis in an IGF-independent manner remains largely unknown, the phenomenon indicates the potential utility of truncation mutations to enhance molecular function as well as provide structural insights. Here, we made three truncations of REIC/Dkk-3 by deleting two cysteine-rich domains (CRDs) that control protein folding and secretion [8]; the loss of which may therefore lead to augmentation of ER stress.

Various carriers for delivering genes have been investigated. They can be divided into two main groups; viral carriers and cationic molecular carriers that form electrostatic interactions with DNA [9]. Although the *in vivo* expression levels of synthetic molecular gene vectors are lower than viral vectors and gene expression is transient, these vehicles are likely to show several advantages including safety, low-immunogenicity, and capacity to deliver large genes and large-scale production at low-cost. The two leading classes of synthetic gene delivery systems that have been most investigated are cationic lipids and cationic polymers.

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In terms of safety, cationic polymers appear the most promising for clinical use [10].

Here, we investigated which truncated form of REIC/Dkk-3 induced apoptosis most effectively, and then successfully established a non-viral cancer gene therapy system by coupling a genetically engineered REIC/Dkk-3 with a biodegradable polymer.

Materials and methods

Cell culture and transfection. PC3 prostate cancer cell line was purchased from ATCC and maintained with Hams F12 supplemented with FBS 10%. PC3/Luc cells which are stably transfected with luciferase gene were purchased from (SUMMIT Pharmaceutical International Corporation, Tokyo, Japan). For plasmid transfection cells were plated on a 12 well dish and transfection was performed when cells become 60% confluent using TransIT®-Keratinocyte (Mirus Bio Corporation. Madison, WI, USA) as transfection reagent as previously described [11].

Apoptosis assay. To examine *in vitro* apoptosis induction after the treatments, cells were seeded in flat-bottom 6-well plates and incubated for 24 h. After 48 h plasmid transfection, Hoechst 33,342 stock solution was added into the medium to the 2 μg/ml concentration and the cells were incubated in the dark for 10 min. Hoechst 33,342 is an intercalating dye that allows determination of total chromatin quantity variations and the degree of chromatin condensation. Using fluorescence microscopy, apoptotic cells were identified by the presence of highly condensed or fragmented nuclei. Apoptotic cells were counted at 3–5 different fields of the microscopic observation. One hundred cells were judged under one field.

Western blot and antibodies. Cell lysates were prepared using M-PER Mammalian Protein extraction Reagent (Pierce, Rockford, IL, USA). Gel electrophoresis and Western blot analysis were performed under conventional conditions. Each specific antibody binding was detected with horseradish peroxidase-conjugated respective IgG antibodies with enhancement by ECL Plus Western Blotting Detection System (GE Healthcare, Amersham Place, UK). Rabbit anti-human REIC/Dkk-3 antibodies were produced in our laboratory [5]. The other first antibodies used here were following: rabbit anti-human stress-activated protein kinase/JNK antibody (Cell Signaling Technology, Beverly, MA), rabbit anti-human phospho-stress-activated protein kinase/pJNK (Thr^{183}/Tyr^{185}) antibody (Cell Signaling Technology), rabbit anti-human CHOP (abcam), rabbit anti-human Bip (Cell Signaling Technology), mouse anti-human α-tubulin antibody (Sigma, St. Louis, MO, USA), anti-mouse GFP antibody (Santa Cruz).

Adenovirus production. REIC/Dkk-3 adenovirus were produced and propagated as described previously [5].

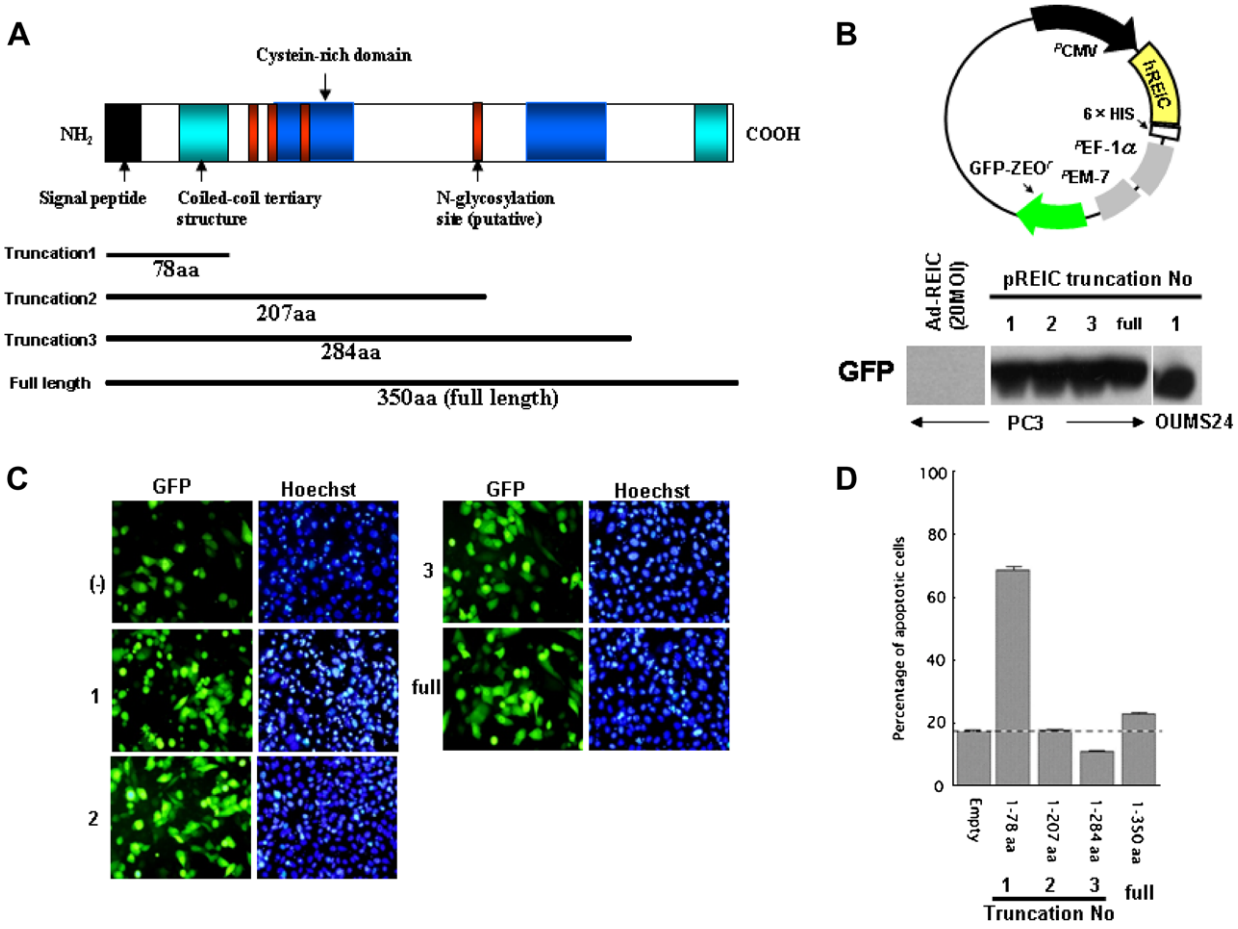

Fig. 1. (A) Scheme of putative REIC/Dkk-3 structural motifs. Using the Ensemble Genome Research Database (National Human Genome Research Institute), we investigated putative structural motifs in REIC/Dkk-3. REIC/Dkk-3 contains two coiled-coil tertiary motifs, 2 cysteine-rich domains (CRDs), and four putative N-glycosylation sites. (B) Plasmid map used in this study. We made and used constructs where each truncation or full length DNA of REIC/Dkk-3 was subcloned into the MCS in pTracer2, which contains a gene encoding a GFP/zeocin fusion protein driven by a EF-1α/EM-7 promoter (upper panel). Western blot analysis of GFP expression (lower panel). To investigate transfection efficiency of pTracer2, GFP expression was examined for each truncation or full length cDNA of REIC/Dkk-3. (C) Apoptosis assays in PC3 cells transfected with each truncation variant or full length cDNA of REIC/Dkk-3. Fluorescence microscopic study showed that the largest number of apoptotic PC3 cells (highly condensed or fragmented nuclei) was detected in variant #1-transfected PC3 cells among the five groups examined. (D) Percentage of apoptotic PC3 cells. Error bar indicates S.E.

ER stress evaluation in vitro. Two micro gram of plasmid pERAI was used for IVIS imaging of ER stress [12]. Two micro gram of #1 or full length REIC/Dkk-3 DNA was co-transfected with pERAI construct. Twenty four hours after co- or single-transfection of pERAI, cells were treated 3 micro molar of thapsigargin (TPG, Sigma–Aldrich) or Ad-REIC (20 MOI). ER stress was monitored 24 h after treatment of TPG or Ad-REIC.

In vivo transgene expression. Two million of PC3 prostate cancer cell line (50 μl) in mixture with Matrigel (50 μl) were directly inoculated into the right flank of an 8 weeks Balb/C nude mouse. After 5 days tumor formation was observed. Twenty five micro gram of pGL3 control vector encoding luciferase gene (Promega) was directly injected into the tumor in mixture with biodegradable cationic polymer, CarriGene (25 μg of pGL3 control vector in mixture with CarriGene 25 μl, 5% glucose medium was used as vehicle. Nitto Denko Technical Corporation, San Diego, CA, USA). IVIS evaluation was done after 24 h of plasmid injection. All the animal experiments were done according to a guideline determined in our University.

IVIS monitoring of in vivo ER stress. Three days after tumor formation to be observed, 25 μg of pERAI was inoculated in mixture with CarriGene 25 μl and 5% glucose medium into each mice. After 24 h, mice received a second pERAI injection. IVIS evaluation was conducted after 24 h of the second treatment.

In vivo therapeutic experiments. Five million of PC3/LuC prostate cancer cell lines were injected subcutaneously in mixture with Matrigel. Twenty four hours after cell injection, mice were evaluated by IVIS and first dose for treatment of #1 was performed. pDsRed2 (clontech) was used as control plasmid. Five mice of each group were analyzed. The light of emission in each mouse was measured by IVIS system (photon/count) at day-7 and -14 after cell implantation.

Results

Truncation No.1 of REIC/Dkk-3 (variant #1) efficiently induced apoptosis in PC3 cells

First, we aimed to discover a minimum element of functional REIC/Dkk-3. Based on a structural prediction of REIC/Dkk-3 in the Ensemble Genome Research Database, we designed three truncations of REIC/Dkk-3 by deleting the cysteine-rich domains (CRD) one by one (Fig. 1A), and subcloned each variant into pTracer2-CMV (Invitrogen) to develop $^{1-78}$REIC/Dkk-3-#1, $^{1-207}$REIC/Dkk-3-#2, and $^{1-284}$REIC/Dkk-3-#3 (Fig. 1B). When the variants were transfected into PC3 cells, the GFP expression levels were approximately equal to each other (Fig. 1B, Western blot) and GFP positive ratios were more than 80% under microscopic observation (Fig. 1C), suggesting successful and equal efficiency of transfection despite the different lengths of the DNA constructs.

Using these plasmids, we investigated apoptosis in PC3 cells. Even naked DNA transfection of variant #1 was able to potently induce apoptosis in PC3 cells (~70%), while variants #2 (207aa) and #3 (284aa) appeared to lose the ability to induce apoptosis (Fig. 1C and D). Neither the truncated nor full length forms were able to induce apoptosis in human fibroblast (data not shown). These results demonstrated that variant #1 induced apoptosis more strongly than the other truncated forms and the full length REIC/Dkk-3. This

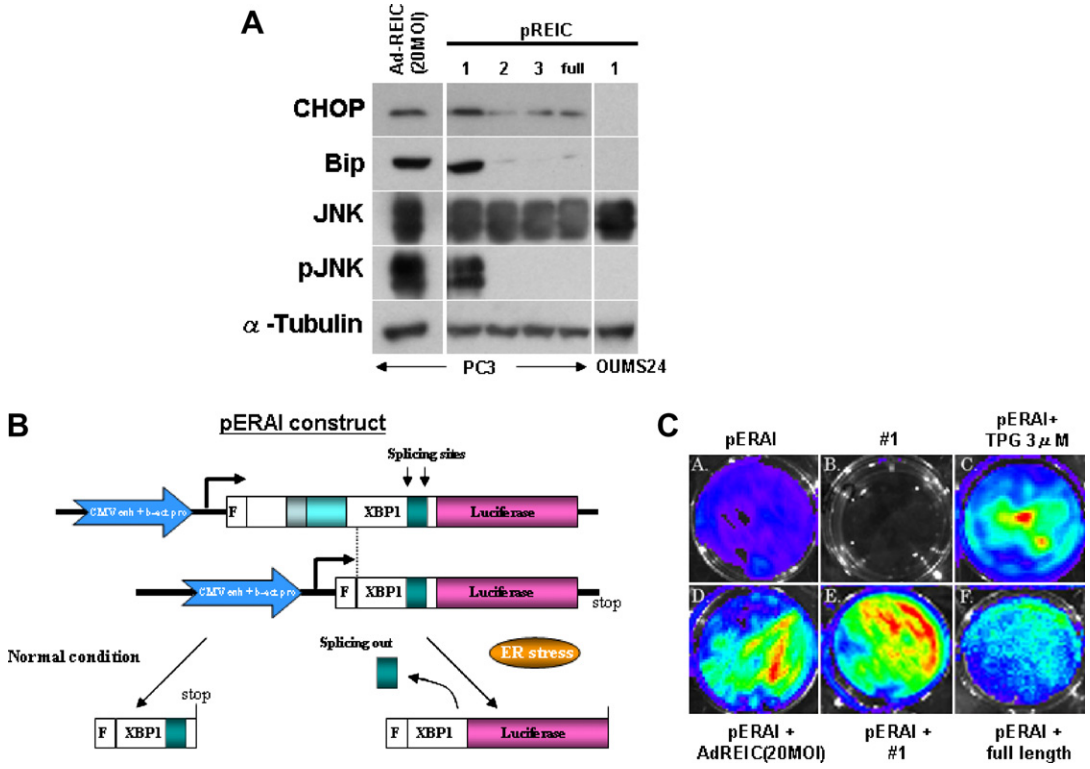

Fig. 2. (A) Western blot analysis of ER stress marker molecules (CHOP and Bip) and apoptosis pathway (JNK and pJNK) in PC3 cells transfected with either Ad-REIC (20MOI) or each truncated or full length cDNA of REIC/Dkk-3. pJNK: phosphorylated JNK. (B) Schematic diagram of ER stress-activated indicator (ERAI) construct. IRE-1 induces activation of its own RNase domain during ER stress. XBP-1 mRNA is a target of unconventional splicing by IRE-1α. In human cells, a 26-nucleotide (nt) intron (nt 531–556) of XBP-1 mRNA is spliced out during ER stress, leading to a frame shift. The spliced XBP-1 mRNA is translated into the mature XBP-1 protein, which acts as a transcription factor in UPR induction. Under conditions of ER stress, the spliced mRNA is translated into an XBP-1-luciferase fusion protein in the ERAI construct. (C) IVIS analysis of ER stress in PC3 cells transfected with either Thapsigargin (TPG), Ad-REIC (20MOI), variant #1 or full length cDNA of REIC/Dkk-3. Thapsigargin (TPG), ER stress inducer, was used as a positive control.

form therefore can potentially be used specifically for killing cancer cells, in the absence of the other functions of the full length protein.

Naked DNA transfection of variant #1-induced ER stress more strongly than full length REIC/Dkk-3 in PC3 cells

Because the intra-molecular disulfide bond in the CRD is crucial for protein folding and secretion, disrupting the CRD was predicted to lead to accumulation of unfolded REIC/Dkk-3 protein and enhance ER stress. Since ER stress is thought to be a major molecular mechanism through REIC/Dkk-3-induced apoptosis [6], a CRD-deleted construct, such as variant #1, was expected to increase its ability to enhance ER stress. As shown in Fig. 2A, expression of ER stress molecules (Bip and CHOP) was substantially increased in variant #1-transfected cells. JNK phosphorylation, which is a crucial event controlling REIC/Dkk-3-induced apoptosis, subsequently occurred in variant #1-transduced cells, as well (Fig. 2A). No significant change in ER stress molecules and JNK phosphorylation was detected in variant #1-transfected normal fibroblast cells (OUMS-24). Furthermore, we determined the increased ability of variant #1 to induce ER stress by double transfection with the pERAI construct, which can monitor ER stress via the intensity of luciferase activity (Fig. 2B). As shown in Fig. 2C, ER stress was significantly increased in variant #1-transfected cells and its intensity appeared to be as strong as that by 20MOI of REIC/Dkk-3 adenovirus (Ad-REIC). These results suggest that ER stress is a key molecular event for apoptotic induction in cancer cells by naked DNA transfection of variant #1 as well as by Ad-REIC infection.

Polymer-mediated variant #1 gene transfer reduced subcutaneous PC3 tumor

For *in vivo* gene transfer, we used a cationic biodegradable polymer because it is safe and can be used several times in the same individual. Prior to therapeutic experiments, we examined the polymer-mediated *in vivo* trans-gene (luciferase) expression in subcutaneous PC3 tumors inoculated in nude mice. As shown in Fig. 3A, certain gene products following injection of pGL3 control vector (encoding luciferase gene) into tumor were assured by the IVIS system, indicating availability of the polymer-mediated *in vivo* gene transfer. Next, we checked *in vivo* ER stress using the pERAI construct. Consistent with the *in vitro* results, IVIS analysis showed much stronger IVIS signals detected in variant #1-transfected subcutaneous tumors than in those transfected with full length DNA (Fig. 3B), demonstrating definitive ER stress evoked in the variant #1-transfected tumor. Finally, therapeutic experiments revealed that multiple injections of polymer-conjugated

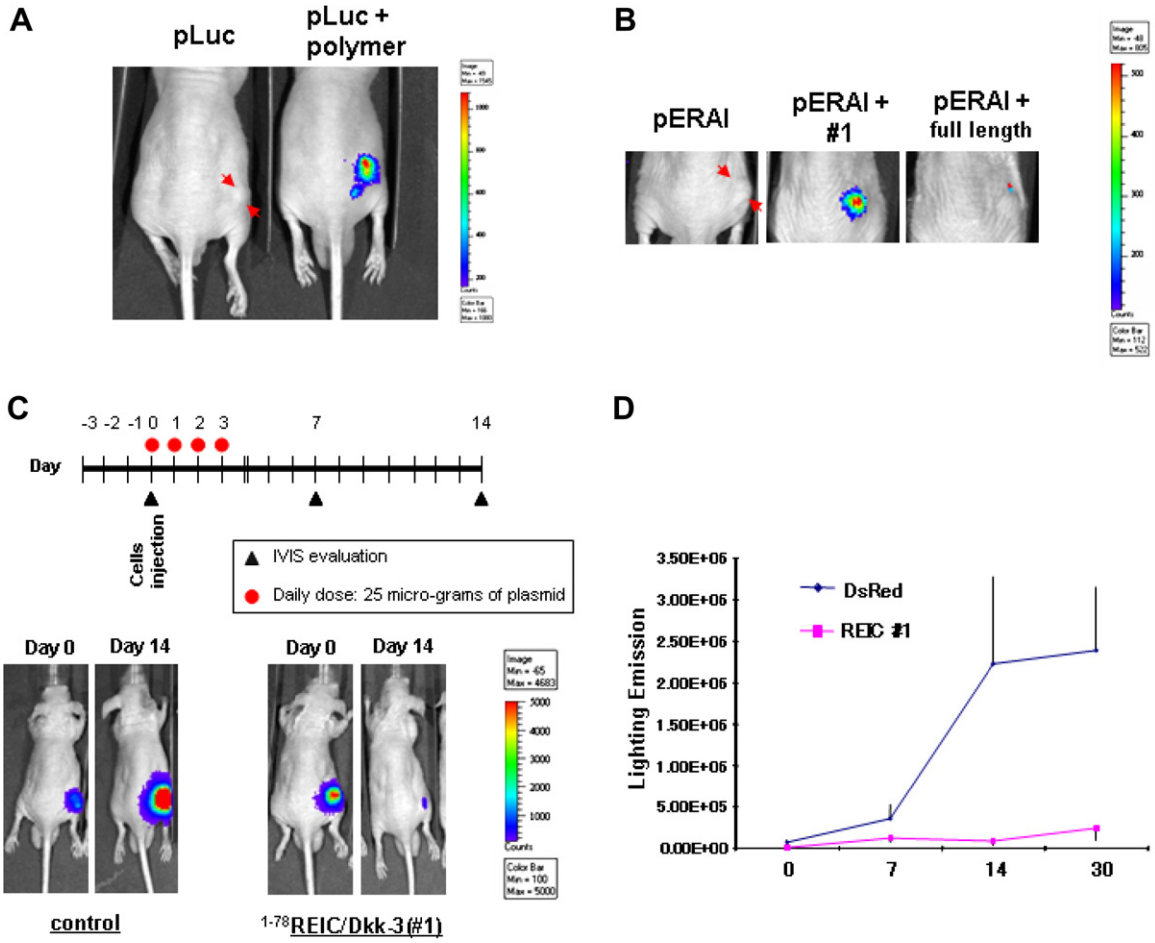

Fig. 3. (A) IVIS analysis of luciferase gene expression in PC3 subcutaneous tumor in which pGL3 control vector encoding luciferase gene was injected with or without biodegradable polymer. Red arrows indicate subcutaneous tumors. (B) IVIS analysis of ER stress in PC3 subcutaneous tumor in which REIC/Dkk-3 truncation variants #1 or full length were co-injected with the ERAI construct using a biodegradable polymer. (C) Timetable of *in vivo* experiments and IVIS analysis of variant #1-mediated anti-tumor effects in PC3 subcutaneous tumor at Day 14. PC3/Luc cells stably transfected with a luciferase gene were inoculated in the right flank of nude mice. Polymer-conjugated variant #1 naked plasmid was directly injected into the PC3/Luc subcutaneous tumor as shown in the timetable. As a control plasmid in this experiment, we used pDsRed2. Tumor regression was evaluated by luminescence from the PC3/Luc tumors at Day 7, 14, and 30. (D) Statistical analysis of luminescence from PC3/Luc tumors. Error bar indicates S.E.

variant #1 significantly reduced subcutaneous PC3/Luc tumors, which were derived from PC3/luc cells, relative to the control group (Fig. 3C and D). No systemic side effects were observed in variant #1-treated mice. Taken together, these results indicate that the biodegradable polymer-mediated variant #1 gene transfer may serve as a promising basis for cancer gene therapy.

Discussion

Given that ER stress is highly correlated with the promotion of apoptosis, in this study we engineered a minimal active form of REIC/Dkk-3 by deleting cysteine-rich domains (CRDs) that were still able to efficiently provoke ER stress and induce apoptosis. An N-terminal 78 amino acid (aa) truncation of REIC/Dkk-3 ($^{1-78}$REIC/Dkk3; variant #1), created by deleting two CRDs, was shown to considerably up-regulate ER stress and exhibit potent apoptotic effects. Interestingly, variant #2, which removed only the second CRD had no ability to induce ER stress or apoptosis, indicating the possibility that deletion of both CRDs is essential for the promotion of ER stress in our experimental system. However, the particular roles of the first and second CRDs in protein folding and secretion and whether losing only the first CRD as opposed to both CRDs forms the basis for increased ER stress, remains unclear. Further analysis, including selective cysteine mutagenesis in the CRDs, will be necessary to provide a better understanding of the role of the CRD domains.

ER stress is a key underlying event in REIC/Dkk-3-induced apoptosis [6]. Beyond their capacity for glycosylating or folding proteins in the ER, several ER stress molecules can be up-regulated to maintain ER homeostasis in the so-called unfolding protein response (UPR) [13]. Whether ER stress contributes to survival or death of cancer cells remains unclear [14]. Many studies have detected UPR activation in a variety of tumor types. Some recent studies have demonstrated that interference with the activation of different arms of the UPR or alteration of the levels of the ER molecular chaperone GRP78/BiP, a master regulator of ER function and the UPR, can inhibit tumor growth *in vivo* [15]. Therefore, to disrupt ER homeostasis and activate programmed cell death (PCD) pathways, severe ER stress must be induced. The gene transfer of polymer-conjugated variant #1 was effective in provoking severe ER stress strongly enough to activate the PCD pathway. Hence, a combination therapy stimulating the ER-stress mediated apoptotic pathways should reduce the number of required injections. It is therefore reasonable, as our previous report suggested, to combine heat shock protein 70 (Hsp70) inhibitor with REIC/Dkk-3 in cancer gene therapy [16]. Further analyses of REIC/Dkk-3-induced ER stress may yield even better anti-cancer strategies using REIC/Dkk-3.

Direct multiple injections of the polymer-conjugated naked DNA of variant #1 into subcutaneous PC3 tumor showed considerably more robust anti-tumor effects compared with the control group. We successfully took advantage of its low-immunogenicity by repeating injections over a number of days *in vivo*. Although in this study we did not directly compare anti-tumor effects between single injection of Ad-REIC and multiple injections of polymer-conjugated variant #1, anti-tumor effects of both strategies are likely to be comparable. In future, non-viral gene delivery strategies such as the cationic polymer-based technique used here may become dominant in clinical gene therapy applications.

In conclusion, we successfully established a non-viral cancer gene therapy system by combining an engineered REIC/Dkk-3 and a biodegradable polymer. We suggest this strategy may be a viable alternative to viral gene therapy.

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