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RESEARCH ARTICLE

A novel targeting modality for renal cell carcinoma: human osteocalcin promoter-mediated gene therapy synergistically induced by vitamin C and vitamin D_3

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Abstract

Background Advanced renal cell carcinoma (RCC) frequently develops skeletal metastasis and is highly resistant to conventional therapies. We hypothesized that the osteocalcin (OC) promoter may be a promising gene delivery system for RCC targeted gene therapy because osteotropic tumors gain osteomimetic properties and thrive in the new environment by exhibiting a bone-like gene expression profile. Human OC (hOC) expression is highly regulated by vitamins and hormone. In the present study, we tested the feasibility of vitamin-regulatable hOC promoter for RCC-specific transcriptional targeting, and examined the anti-tumor effect of vitamins C and D_3 with hOC-based adenoviral vectors towards RCC.

Methods Real-time reverse transcriptase-polymerase chain reaction measured OC expression induced by vitamins C and D3, either alone or in combination, in RCC and human renal epithelial cells (HRE) normal renal epithelial cell lines. The RCC-cytotoxic effects of concomitant vitamins and hOC promoter-based adenoviral vectors, Ad-hOC-TK and Ad-hOC-E1, were evaluated in both cell culture and a xenograft murine model.

Results We found that high doses of vitamin C induced H_2O_2-dependent apoptosis in RCC but not HRE. Treatment of RCC cells with combined vitamins C and D_3 treatment significantly increased OC promoter activity compared to single reagent treatment. Combined vitamin therapy reduced tumor size (85%) and complete tumor regression occurred in 38% of mice co-administrated Ad-hOC-E1.

Conclusions The results obtained in the present study demonstrate that vitamins C and D_3 synergized with the anti-tumor effects of therapeutic genes driven by hOC promoter through direct cytotoxicity as well as transcriptional targeting. This combined gene therapy provides a promising modality for advanced RCC targeted therapy. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords adenoviral vectors; gene therapy; osteocalcin promoter; renal cell carcinoma; vitamin C; vitamin D_3

Introduction

Human renal cell carcinoma (RCC) is the most common, malignant form of kidney cancer that arises from renal epithelium. The age-adjusted incidence of this disease has been rising by 3% per year during the past five decades [1].

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Approximately 57 760 new cases of RCC and 12 980 deaths are expected to have occurred in the USA in 2009 [2]. Up to one-third of patients with RCC have metastases at presentation [3], and approximately 40% of patients treated for a localized tumor develop recurrence [4]. Despite improvements in the management of localized RCC, treatment of advanced RCC with systemic therapies or surgical intervention has been largely unsuccessful. Advanced RCC patients have an extremely poor outcome with an estimated median survival of less than 1 year [5]. Thus, the development of new agents with more effective anti-tumor activity, in particular targeting the metastatic phase of RCC, merits a high priority in the treatment of advanced RCC.

Gene therapy has been identified as the most promising treatment option for metastatic cancers [6]. Transcriptional regulation of transgene expression using tumor- or tissue-specific promoters within adenoviral vectors has already been attempted to treat tumors [7,8]. However, only a limited number of promoters that restrict gene expression to RCC have been studied [9–11] because clinically defined RCC tumor markers whose promoter is highly active in tumors but either silent or active at very low background levels in normal kidney cells are not available. Therefore, the development of a novel inducible promoter system that allows reliable and controllable transactivation of ectopic gene expression in restricted tissue or cell types by administration of inducing agents is essential for the success of a RCC targeted gene therapy that does not induce serious kidney damage.

Osteocalcin (OC) is a major noncollagenous bone protein, which is deposited onto bone matrices at the time of bone mineralization. OC binds to the extracellular matrix and acts as a chemoattractant for bone-resorbing cells which maintain bone mineral homeostasis [12]. Bone-specific transcription of the OC gene is regulated principally by the Runx2 transcription factor that binds to the osteocalcin-specific elements OSE1 and OSE2, which are located approximately 50 and 140 bp upstream of the transcriptional start site, respectively [13]. OSE2 site is also required for the activation of OC by vitamin C (ascorbic acid) [14,15]. Other important transcriptional elements include the OC box and hormone receptor binding sites, which are both positively and negatively regulated by a number of vitamins and hormones [16]. Rat and human OC, but not mouse OC gene expression, is regulated by a vitamin D responsive element (VDRE) [16,17] recognized by the vitamin D_3 receptor (VDR) complex upon ligand (1α,25-dihydroxyvitamin D3) activation [18,19]. The finding that OC gene is expressed almost exclusively in differentiated osteoblasts and osteotropic tumors, including osteosarcoma [20] and ovarian, lung, brain and prostate cancers [21], has led to the development of OC promoter-mediated targeted gene therapy for the treatment of patients with bone disorders [22,23] or tumor metastasis to the skeleton [21,24–27]. We have previously characterized an approximately 800 bp of human osteocalcin (hOC) promoter, which contains three regulatory elements, OSE1, OSE2 and VDRE [28]. Its activity can be highly induced by vitamin D_3. When the hOC promoter regulated adenoviral *E1A* and *E1B* gene expression in a bi-directional manner, vitamin D-enhanced viral replication was observed in androgen-independent and highly metastatic prostate cancer cell lines [29]. Similar to prostate cancer, RCC frequently metastasizes to the skeleton in the later stages of the disease [30]. This observation suggests that hOC promoter-based expression vectors combined with transcriptional inducers may provide a novel inducible gene delivery system for the treatment of human RCC.

In the present study, we showed that treatment of human RCC cells with hOC promoter and its inducers vitamin C and vitamin D_3 together significantly increased OC expression compared to single agent treatment. The triple agent treatment had no effect on normal renal epithelial cells, which have an undetectable basal level of OC promoter activity. The combination of vitamins C and D_3 synergized with the anti-tumor effects of therapeutic genes driven by hOC promoter on cultured RCC cell lines and established RCC tumors in immunodeficient mice. The results obtained provide the first *in vivo* demonstration of the efficacy and safety of triple combination therapy of the hOC promoter-based adenoviral vectors, vitamin C and vitamin D_3 for the treatment of human RCC.

Materials and methods

Cell lines and cell culture

The established human renal cell carcinoma cell lines, RCC29, RCC45, RCC6 and RCC42, were described previously [10] and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen, Grand Island, NY, USA). Normal human renal epithelial cells (HRE) purchased from Lonza (Rockland, ME, USA) were maintained in renal epithelial cell growth medium in accordance with the manufacturer's instructions (Lonza).

Reagents and adenoviral vectors

Vitamin D_3 analog (Ro 25–9022) was provided by Roche (Nutley, NJ, USA). Ethanol was used as the vehicle control for vitamin D_3. L-ascorbic acid and catalase were purchased from Sigma Aldrich (St Louis, MO, USA). The adenoviruses, Ad-hOC-TK and Ad-hOC-E1 used in the present study, were produced and described previously [25,29]. Ad-hOC-TK, a replication-defective adenovirus, expresses herpes simplex virus thymidine kinase under the control of a 3.9-kb human OC promoter. Ad-hOC-E1 is a conditional replication-competent adenovirus containing a single bidirectional 800-bp human OC promoter to drive both early viral *E1 A* and *E1B* genes.

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Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Cells were treated with 5 nM vitamin D_3 analog (Ro 25–9022) for 48 h. RNA was extracted by using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and first-strand cDNA was synthesized by using 1 μg of total RNA with Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen, Grand Island, NY, USA) in accordance with the manufacturer's instructions. The primer sequences for hOC were 5′-ACACTCCTCGCCCTATTG-3′ (forward) and 5′-GATGTGGTCAGCCAACTC-3′ (reverse) and for GAPDH were 5′-ACCACAGTCCATGCCATCA-3′ (forward) and 5′-TCCACCACCCTGTTGCTGT-3′ (reverse).

Real-time quantitative PCR

Cells were treated with 5 nM vitamin D_3 analog (Ro 25–9022) and 0.15 mM vitamin C alone or combination for 48 h. Quantitative RT-PCR was performed using the LightCycler 480 TaqMan master kit with Universal ProbeLibrary probe (Roche Applied Science, Mannheim, Germany). The primers and probes were designed by a web-based Assay Design Center (http://www.universalprobelibrary.com). The real-time PCR reaction was conducted in accordance with the manufacturer's instructions, consisting of a denaturation step (10 min) and 55 cycles of amplification (95 °C for 10 s, 60 °C for 10 s followed by single fluorescence acquisition at 72 °C for 10 s). The relative gene expression of specific target in each group was represented as $2^{-\Delta CT}$, the ΔCT is determined by subtracting the average housekeeping gene HSPCB C_t value from the average target gene value.

Apoptosis detection by annexin V binding assay

Annexin V binding assays were performed by using an Annexin V-FITC Apoptosis Detection kit in accordance with the manufacturer's instructions (Sigma Aldrich). Briefly, RCC cells were exposed to various concentrations of vitamin C for 24 h. Both floating and adherent cells were collected. After a rinsing step with phosphate-buffered saline (PBS), cells were resuspended in binding buffer that contained FITC-conjugated annexin V and propidium iodide (PI) for 15 min and subjected to flow cytometry analyzed with a FACScan (Becton Dickinson, Mountain View, CA, USA). Results were integrated with the CellQuest software (Becton Dickinson) for calculation of percentage cells with apoptosis per group.

In vitro cytotoxicity assays

For vitamin C induced cytotoxicity, cells seeded on 24-well plates were incubated with vitamin C (L-ascorbic acid) at a concentration of 0–200 μg/ml in the presence or absence of 100 U of catalase. For adenoviral vector-induced cytotoxicity, cells were infected with adenoviral vectors at a range of multiplicity of infection (MOI). After 2 h of adsorption, the virus-containing medium was replaced with fresh medium. After 24 h, cells infected with adenoviral vectors were incubated with 5 μM vitamin D_3 or 150 nM vitamin C, or both. An additional 10 μg/ml ganciclovir was used as the prodrug for Ad-hOC-TK-infected cells. After 7 days of treatment, the viable cells were detected by crystal violet staining. Each experiment was carried out either in duplicate or triplicate.

Human RCC xenograft model

Institutional guidelines and an Animal Research Committee-approved protocol were followed for mouse studies. Male nu/nu mice aged 5–6 weeks old were obtained from Charles River (Wilmington, MA, USA). Subcutaneous tumors were established by injecting 2×10^6 RCC42 cells into the bilateral flanks of mice. When tumors reached 100 mm^3, four treatment groups were randomly assigned ($n = 8$ in each group): PBS controls, vitamin C (15 g/l in drinking water), Ad-hOC-E1 (2×10^9 pfu, i.v. injection with single dose) plus vitamin D_3 (4 ng/dose, i.p. injection every other day for 3 weeks) and Ad-hOC-E1 plus vitamin C and D_3. Vitamin D_3-treated mice were fed a sterilized calcium deficient diet (ICN Research Diets, Costa Mesa, CA, USA). Tumors were measured weekly with calipers. Volumes were calculated by the formula: volume $= 0.5236 \times \text{width}^2 \times \text{length}$. Data were expressed as the fold of tumor volume increase, obtained by assessing tumor size relative to the initial size at the time of treatment. The mice were sacrificed 6 weeks after treatment. Tumors were dissected, fixed in formalin, and subjected to histopathological examination.

In situ analysis of apoptotic cells in tissues

Apoptosis was evaluated using the Apo-BrdU-IHC In Situ DNA Fragmentation Assay Kit (BioVision, Inc., Mountain View, CA, USA) as described previously [31]. Briefly, paraffin-embedded tumor sections were dewaxed and permeabilized with proteinase K for 20 min. The DNA strand breaks were labeled with BrdU in a terminal deoxynucleotidyl transferase reaction mixture at 37 °C for 1 h, and detected with anti-BrdU-Biotin conjugate with diaminobenzidine in accordance with the manufacturer's instructions. The samples were counterstained with 1% methyl green to show viable cells. Cells in which the nuclei were clearly dark brown were considered to be apoptotic cells.

Statistical analysis

Differences between treatment groups were analyzed using Student's t-test and two-tailed distribution. $p < 0.05$ was considered statistically significant.

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Results

Vitamin C synergized with vitamin D_3 to induce osteocalcin expression in human RCC cells

To assess whether human OC promoter-based gene therapy combined with vitamin D_3 may be useful for the treatment of human renal cancers without affecting normal kidney cells, semi-quantitative RT-PCR was performed to compare the basal and vitamin D_3-induced OC expression in human normal and malignant renal epithelial cell lines (Figure 1A). The expression of OC mRNA can be detected clearly in MG63, a human osteoblastic cell line, under normal culture conditions. This expression was further stimulated by vitamin D_3 analog (Ro 25−9022) treatment, which demonstrated the regulatory activity of vitamin D_3 on OC expression. Both normal renal epithelial cells and renal epithelial cancer cell lines that we tested had very low or undetectable OC mRNA expression. Vitamin D_3 markedly induced OC expression in all tested RCC cell lines excepting RCC45, which showed a relatively lower induction, but caused no change in OC expression of normal HRE cells. We also found that the RCC cell lines and MG63 in which OC was induced by vitamin D_3 expressed VDR, whereas normal HRE cells which had no to minor basal and vitamin D3-induced OC promoter activity lacked VDR expression. Moreover, transfection of RCC cells with VDR-specific targeting siRNA significantly attenuated vitamin D_3-induced OC mRNA expression (see Supporting information, Figure S1). These results suggested that the VDR complex with VDRE in the proximal region of the human OC promoter plays a major role for up-regulating the OC transactivating activity in RCC and bone cell lines.

The vitamin highly induced OC gene transcription in RCC but not in normal HRE was also confirmed by quantitative RT-PCR (Figure 1B). Vitamin D_3 exerted a 26-fold induction in OC expression in RCC42 cells. In addition, vitamin C (L-ascorbic acid) treatment itself induced moderate OC expression (3.8-fold increase) and synergized with vitamin D_3 to reach a more than 80-fold transcriptional induction. However, HRE barely responded to the OC induction by these vitamins, either alone or combined treatment, with less than significant (≥ 2) fold changes in expression compared to cells grown in the normal conditions.

Runx2 is known as a primary bone-related transcriptional regulator in modulating OC expression in osteoblasts [32]. We further determined whether Runx2 played a role in the OC induction by vitamin C and vitamin D_3 by examining the influence of vitamins on Runx2 gene transcription. Consistent with other data obtained in rat and mouse osteoblastic cells [33,34], our quantitative RT-PCR result revealed a 33% reduction of Runx2 transcripts in RCC42 cells after vitamin D_3 treatment (Figure 1C). By contrast, vitamin C enhanced Runx2 gene transcription by 4.6-fold compared to that of untreated cells. This vitamin C-dependent up-regulation of Runx2 was 40% reduced in those cells concomitantly treated with vitamin D_3. Similar to the OC induction, no changes in Runx2 transcription was observed in HRE cells under the vitamin treatment conditions. Taken together, these results suggested that OC gene expression in RCC is differentially regulated by vitamin D_3 and vitamin C and that both pathways functionally interact.

Vitamin C induced apoptosis in RCC but exhibited no toxicity to normal renal epithelial cells

Vitamin C (ascorbic acid) or its derivatives have shown antineoplastic properties against several malignant cell lines [35−37]. We assessed the effect of vitamin C on cultured RCC cells. An *in vitro* cytotoxicity assay (Figure 2) showed a dose-dependent vitamin C-induced cell death in both RCC45 and RCC42 cell lines with 50% inhibitory concentrations (IC_{50}) of 0.3−0.6 mM. By contrast, a higher dose of vitamin C (1.2 mM) promoted rather than inhibited cell growth of normal renal epithelial cells (HRE). This vitamin C-dependent cytotoxicity of RCC cells was completely abrogated by the addition of catalase, an enzyme that degrades hydrogen peroxide (H_2O_2). This finding suggested that H_2O_2 was involved in the vitamin C induced cytotoxic pathway in RCC cells. On the other hand, catalase did not antagonize the vitamin C-induced HRE cell proliferation. To assess whether apoptosis was contributing to the cytotoxic effects of vitamin C towards RCC, we examined annexin V/PI surface staining following treatment with vitamin C or media alone. As shown in Figure 3, vitamin C induced a dose-dependent early (annexin V-positive only) and late (annexin V/PI positive) apoptosis in RCC42 cells. These data demonstrate that cytotoxicity occurred, induced by a high dose of vitamin C, at least in part, through the induction of apoptosis.

Combining vitamin C and vitamin D_3 potentiated the cytotoxicity of hOC promoter-based adenoviral vectors in cultured human RCC cells

Vitamin C and vitamin D_3 together resulted in a significant increase of hOC expression compared to single reagent treatment (Figure 1B). These results raised the possibility that vitamins C and D_3 could synergize with the anti-tumor effects of therapeutic genes driven by hOC promoter on human RCC through the transcriptional induction of exogenous hOC promoter activity. To test this hypothesis, we evaluated two hOC promoter-based adenoviral vectors in the presence or absence of sub-lethal doses of vitamin C (150 nM) and vitamin D_3 (5 nM) for cytotoxicity of RCC42 cells (Figure 4). As shown in

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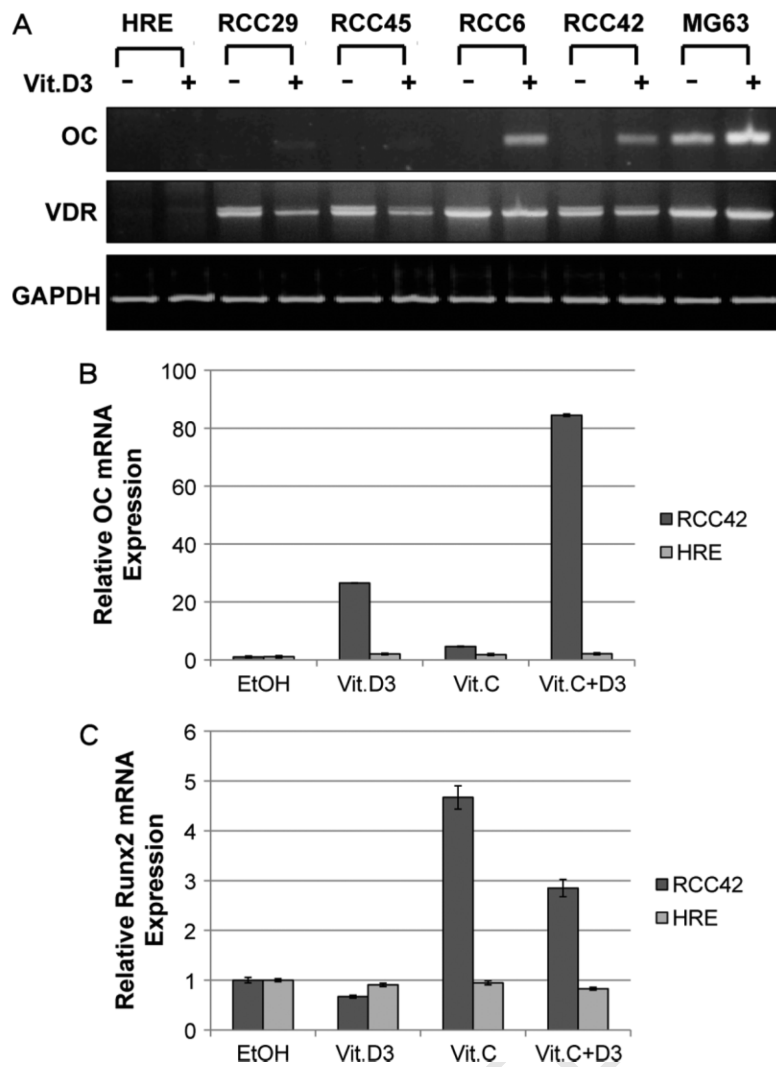

Figure 1. Vitamin D_3 and vitamin C regulated osteoblastic gene expression in human RCC cells. (A). Basal and vitamin D_3-induced OC and VDR mRNA expression in human normal and malignant renal cell lines. RT-PCR was performed using total RNA prepared from a human normal renal epithelial (HRE) cell line, a series of human RCC cell lines (RCC29, RCC45, RCC6 and RCC42), and a human osteoblast cell line (MG63) cultured in the presence or absence of 5 nM vitamin D_3 for 48 h. MG63 cell line was used as a positive control for vitamin D_3 action, and GAPDH was used as the RNA loading control. Quantitative RT-PCR analysis of the induction of (B) OC and (C) Runx2 by vitamin D_3 (5 nM) and vitamin C (0.15 mM), either alone or combination for 48 h, in RCC42 and HRE cells. The data were normalized to housekeeping gene HSPCB expression and presented as fold changes relative to the vesicle control (EtOH)

Figure 4A, a conditional replication-competent Ad-hOC-E1 [29] alone induced cytotoxicity towards RCC42 in a dose-dependent manner. At a MOI of 1, Ad-hOC-E1 alone and combined with vitamin C did not cause significant cell death by day 7. The addition of vitamin D_3 to the Ad-hOC-E1 treatments induced approximately 50% cell lysis (and 25% further when vitamin C was present). Similarly, Ad-hOC-TK/GCV treated cells (Figure 4B) showed evidence of synergistic cytotoxicity by combining vitamin C and vitamin D_3 with Ad-hOC-TK/GCV at a MOI of 30. By contrast, Ad-hOC-TK/GCV and Ad-hOC-E1, either used alone or combined with vitamins C and D_3 had no cell-killing activities in HRE cells (see Supporting information, Figure S2), indicating the selectivity of these agents to malignant cells.

Vitamin C enhanced the anti-tumor effects of systemic Ad-hOC-E1 plus vitamin D_3 therapy on human RCC xenografts in nude mice

To test the therapeutic efficacy of vitamin C and Ad-hOC-E1/vitamin D_3 treatment against human renal cancer cells *in vivo*, nude mice at the age of 6–8 weeks were implanted with RCC42 cells subcutaneously. When tumors were established, groups of tumor-bearing mice were treated with vitamin C only, Ad-hOC-E1 plus vitamin D_3, triple combination of Ad-hOC-E1, vitamin D_3 and vitamin C, or PBS, as the untreated control. RCC42 xenografts were shown to be very aggressive tumors that grew to 35-fold of its initial volume at 6 weeks (Figure 5A). A single tail vein injection of Ad-hOC-E1 combined with vitamin

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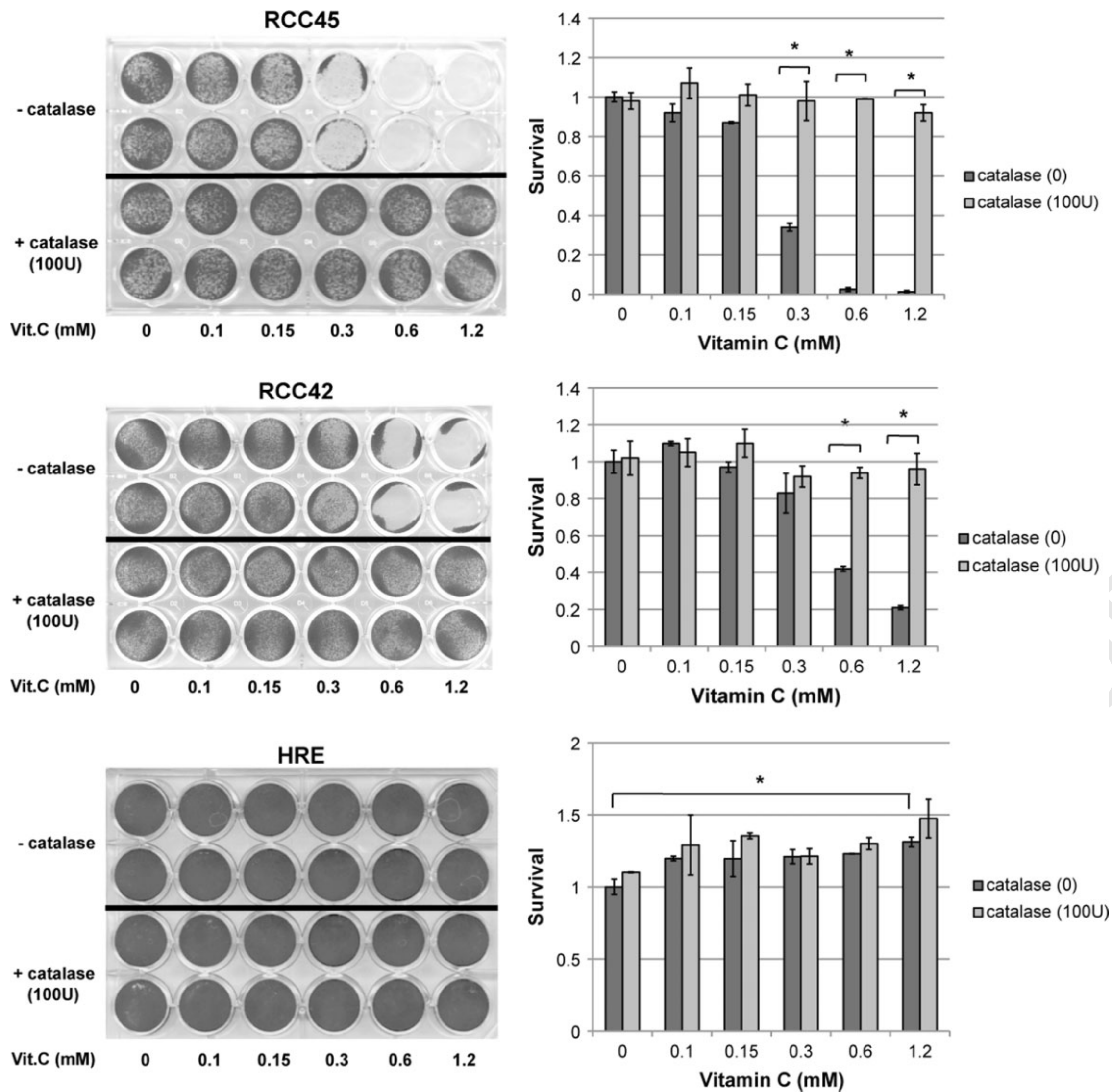

Figure 2. Vitamin C induced H_2O_2-dependent cytotoxicity towards RCC cells. RCC45, RCC42 and HRE cells were cultured in the presence of the indicated concentration of vitamin C with or without the additional catalase (100 U) for 5 days. Cell proliferation was determined by using crystal violet staining (left panel). The relative cell number was assessed by absorbance at 590 nm after staining (right panel). $^*p < 0.05$ significantly different between groups

D_3 administration suppressed tumor growth significantly ($p < 0.05$). Mice drinking water that contained vitamin C also inhibited RCC42 tumor growth with an almost 70% reduction in tumor volume (TV) compared to TV of the untreated group. Triple therapy with Ad-hOC-E1, vitamin D and vitamin C caused the greatest tumor growth retardation because three out of eight animals were completely tumor-free at the end of the treatment period. Histological analysis (Figure 5B; hemotoxylin and eosin) and *in situ* cell death assay (Figure 5B; terminal deoxynucleotidyl transferase dUTP nick end labeling) showed a moderate apoptosis-induced tumor lysis after Ad-hOC-E1/vitamin D_3 treatment. A robust apoptotic response occurred within tumors of animals treated with either vitamin C alone or with triple combination therapy. Taken together, these results demonstrate a synergistic/additive antitumor effect of vitamin C and conditional oncolytic Ad-hOC-E1/vitamin D_3 combination therapy.

Discussion

Metastatic RCC is particularly resistant to classic cytotoxic chemotherapy and hormone therapy [38], and the poor outcomes with cytokine-based therapies leave these patients with an unmet clinical need for alternative therapeutic options. Targeted therapies have been developed to interfere with intracellular signaling involved in cell proliferation, differentiation and angiogenesis [39], thereby inhibiting RCC tumor growth. Sorafenib and sunitinib, the anti-angiogenic tyrosin kinase inhibitors, that target vascular endothelial growth factor and platelet-derived growth factor receptor pathways, have recently been

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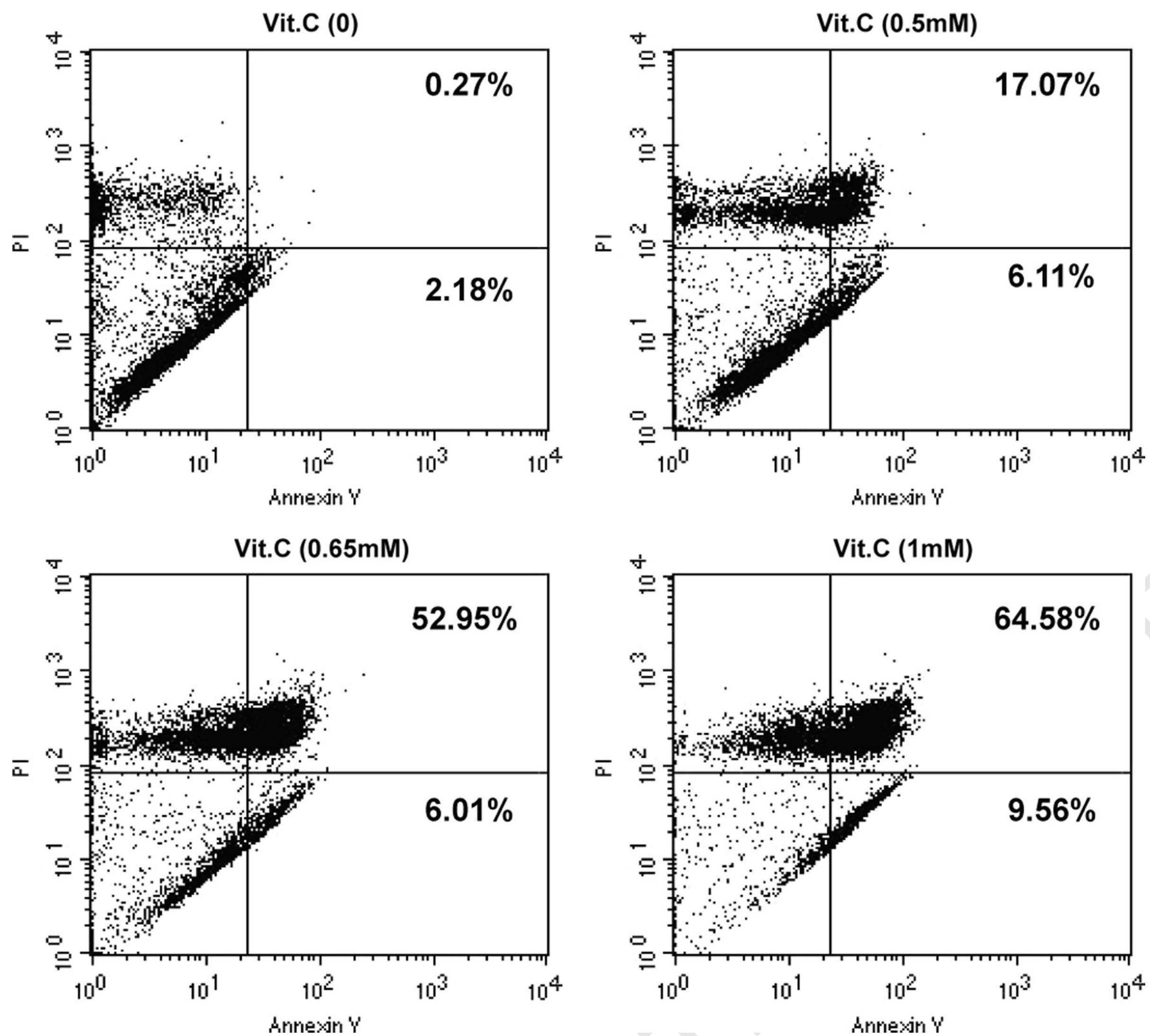

Figure 3. Vitamin C induced RCC cells undergoing apoptosis. RCC42 cells were treated with the indicated concentration of vitamin C for 24 h and then subjected to Annexin V-FITC and PI staining. Apoptotic cells were determined by flow cytometry. Data were presented in the diagrams of Annexin V-FITC (*x*-axis) and PI (*Y*-axis) fluorescence intensity in a representative experiment. Cells in the lower right quadrant indicate annexin-positive, early apoptotic cells. The cells in the upper right quadrant indicate annexin-positive/PI-positive, late apoptotic cells

approved for use as orally administrated agents for the treatment of metastatic RCC [40] and patients with refractory of cytokine therapy [41], respectively. Owing to the pivotal role of mammalian target of rapamycin (mTOR), a serine/threonine kinase in coupling growth stimuli to cell cycle progression [42], two rapamycin derivatives, temsirolimus and everolimus that bind to the FK-506 binding protein-12 and forming a complex specifically with the mTOR complex 1 have undergone clinical evaluation as advanced RCC therapeutics [43,44]. First-line temsirolimus administered to metastatic, poor-prognosis RCC patients significantly prolonged overall and progression-free survival compared to interferon-α [45]. Oral administrated everolimus prolongs progression-free survival in metastatic RCC patients whose disease progressed on or after treatment with sorafenib and sunitinib [46,47]. Beyond the clinical studies demonstrating efficacy, resistance to currently used mTOR inhibitors may potentially arise from positive feedback signaling through rapamycin-insensitive mTOR complex 2 or an mTOR-independent mechanism leading to the downstream PI3K/Akt activation [44]. Adenoviral-based gene therapy that lack of cross-resistance with other treatment options, divergent anti-tumor mechanism and frequently synergistic effects [48] may thus become a promising new adjuvant modality for patients refractory to mTOR inhibitor therapy.

Cancer-specific promoters are useful tools for accomplishing targeted expression: high levels of gene expression in cancer cells are needed to achieve therapeutic efficacy and low expression levels in normal tissues of the liver, lung and kidney are needed to minimize damage that can jeopardize survival of the host. In the present

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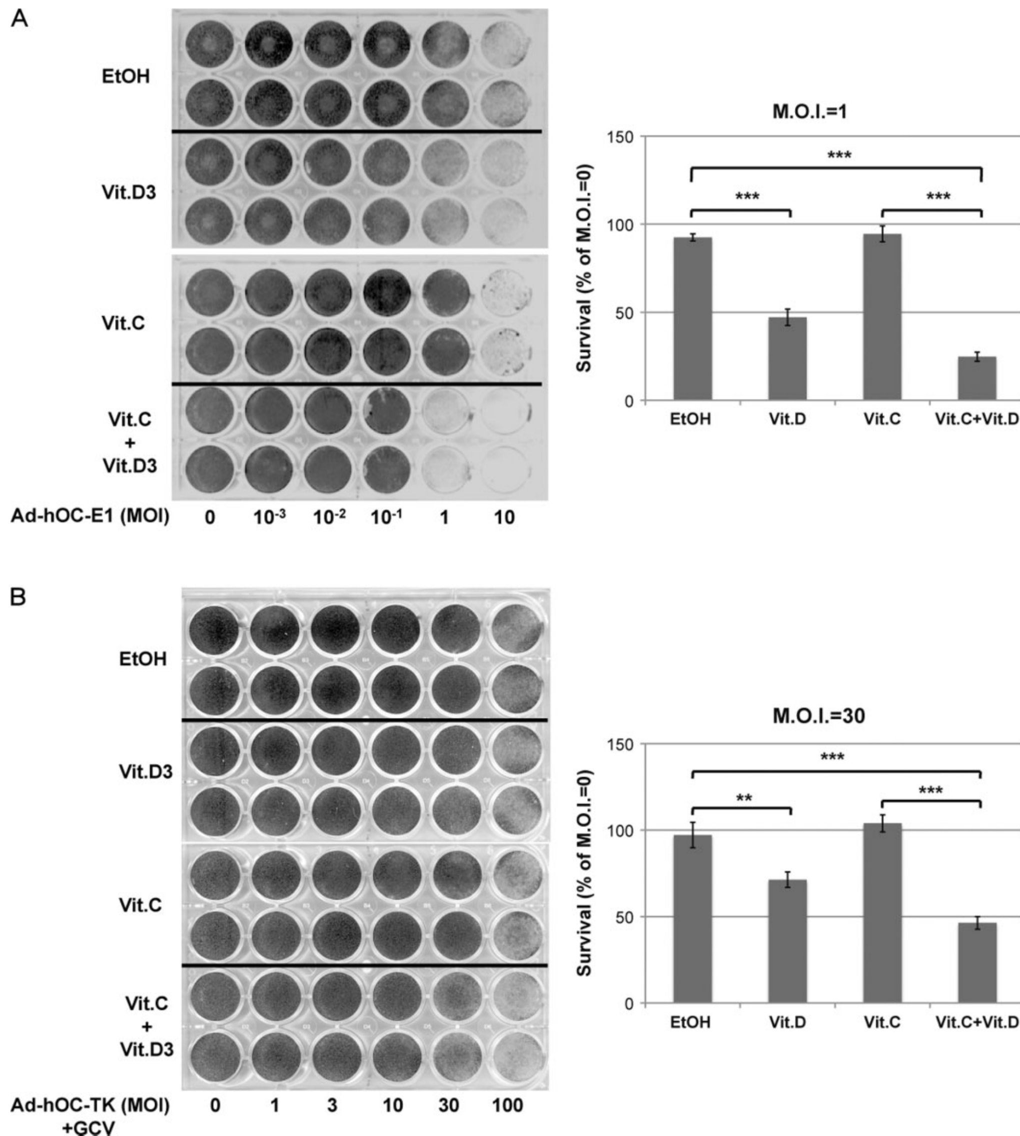

Figure 4. Vitamin C synergized with hOC promoter-based adenoviral vectors to induce RCC cytotoxicity *in vitro*. RCC42 cells infected with (A) conditional replication-competent Ad-hOC-E1 and (B) Ad-hOC-TK at the indicated MOI were cultured in media containing either vitamin D_3 (5 nM) or vitamin C (0.15 mM), or both, for 7 days. An additional 100 µg/ml ganciclovir (GCV) was used as the prodrug for Ad-hOC-TK-infected cells. Cytotoxicity was determined by crystal violent staining (left panel). The relative cell number was assessed by absorbance at 590 nm after staining (right panel). $^{**}p < 0.005$; $^{***}p < 0.001$

study, we characterized the hOC promoter-based expression vector plus vitamins C and D_3 as a regulatable system that is not only capable of finely modulating the expression of gene product to reach the therapeutic range in renal cancer cells, but also maintaining therapeutic gene silence in normal kidney cells to avoid harmful side-effects. The advantages of using vitamins and hOC promoter as a pharmacologically regulated system in human gene therapy are: (i) the ligand activated rather than silenced OC transcription which leads to a rapid induction kinetics; (ii) vitamins can be orally supplemented and they easily penetrate the target tissue; (iii) the vitamin C (L-ascorbic acid) and vitamin D_3 (1α,25-dihydroxyvitamin D_3) are the active metabolic products, which allow a precise calculation of the dosage for effective therapeutic gene expression; and (iv) vitamins have exhibited no potential immunogenicity in humans. The preclinical protocol of the present study has provided a set of conditions that reflects the potential and safe clinical use of the system.

Our quantitative real-time PCR results showed that vitamin D_3 stimulated the basal promoter activity of OC in human RCC but not in normal HRE cells, and this vitamin D_3-dependent OC expression can be further enhanced by vitamin C. The precise mechanisms that cause the differential functions of vitamin D_3 in the normal and malignant renal cells are not fully understood. It has previously been suggested that vitamin D plays an important role in RCC etiology because kidney is a major organ for vitamin D metabolism, activity and calcium homeostasis. Recent studies revealed an association between the genetic variation of vitamin D pathway genes and the

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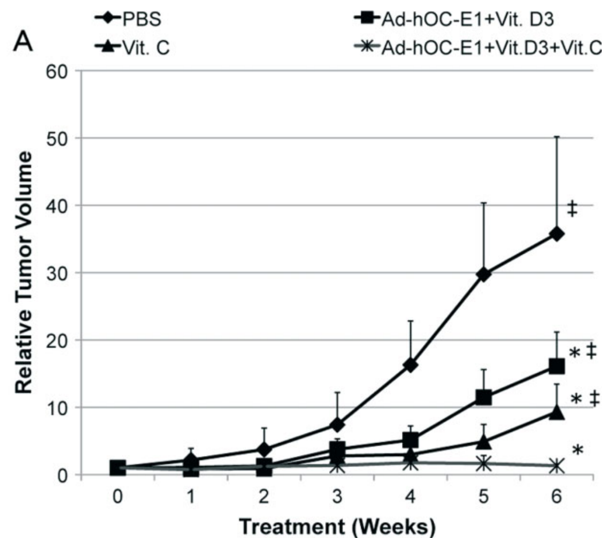

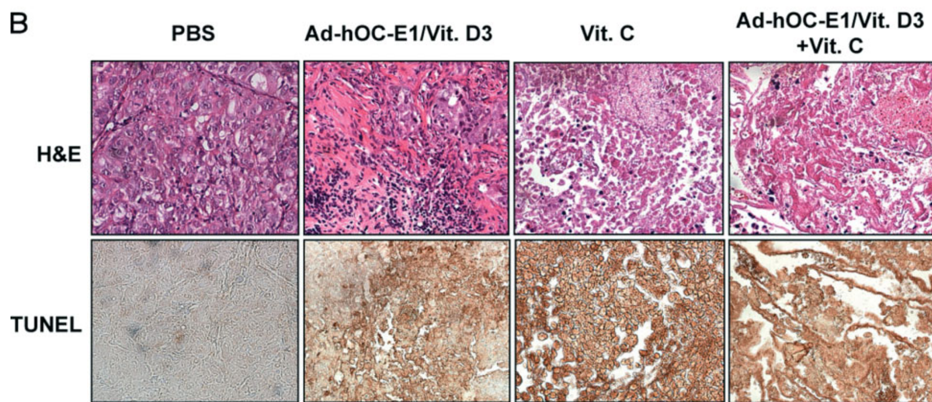

Figure 5. Vitamin C enhanced antitumor efficacy of Ad-hOC-E1 plus vitamin D_3 therapy on RCC42 tumor xenografts in nude mice. (A) Anti-tumor efficacy of oral vitamin C (15 g/l) and systemic AdhOC-E1 (2×10^9 pfu, i.v.) plus vitamin D_3 (4 ng/dose i.p.) therapy, alone or in combination, on human RCC xenografts grown subcutaneously in nude mice. Tumor volume was measured weekly. $^*p < 0.05$ indicates significant differences from the PBS control group, $n = 8$ in all groups. ‡$p < 0.05$ vs. Ad-hOC-E1 + vitamin D_3 + vitamin C group. (B) Pathological analysis of cytopathic effects (hemotoxylin and eosin staining, upper panel) and detection of apoptosis with a terminal deoxynucleotidyl transferase dUTP nick end labeling assay in tumor tissues of tumor-bearing nude mice in different treated groups at the end time point (6 weeks after treatment). Magnification, ×200

increased risk of RCC [49,50]. As a potential mechanism, common variants in VDR and/or RXR genes that are associated with RCC alter the affinity of VDR/RXR complex binding to the regulatory sequences, VDRE, in the promoter of OC, and modulate gene expression. In RCC42 cells, we observed a weak induction in VDR mRNA upon vitamin D treatment, which could serve as a means of signal amplification (see Supporting information, Figure S1). Unlike OC gene, human VDR promoter contains no consensus VDRE [51], suggesting that vitamin D does not directly activate expression of its receptor though traditional steroid hormone receptor-mediated pathways. It has been shown that rapid activation of protein kinase C by vitamin D_3 caused an increase in VDR mRNA expression in rat chondrocytes [52], providing an alternate method for the vitamin D to modulate gene expression. In osteoblastic cells, Runx2 plays a key role in the vitamin D_3-dependent stimulation of the OC gene promoter by recruiting the transcriptional co-activator p300 to the OC promoter and facilitating the subsequent interaction of p300 with VDR upon ligand stimulation [53]. Likewise, vitamin C synergized with vitamin D_3 to activate hOC expression in RCC cells and appeared to involve the upregulated Runx2 that previously has stabilized the binding of the VDR to the VDRE. This tight functional relationship between VDR and Runx2 transcriptional factors in the up-regulation of hOC gene expression strengthens the differentiation of our inducible RCC tumor targeting strategy using triple agents of hOC promoter-based adenoviral vectors with vitamin C and vitamin D_3.

Mechanisms of vitamin C-mediated apoptosis in numerous tumor types have included the down-regulation of iron uptake in neuroblastoma and melanoma cells [54,55], induction of cell cycle arrest in melanoma cells [56], interference with intracellular Ca^{2+} release in hepatoma cells [57], activation of the apoptosis-inducing factor factor in human breast cancer cells [58] and an induction of autophagy in pancreatic cancer cells [59]. The most common theory of vitamin

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C-dependent tumor toxicity relates to its oxidation-reduction properties. In the present study, we showed a growth-promoting effect of vitamin C in normal renal epithelial cells (HRE), in which its antioxidant function may protect cells from oxidative stress. On the other hand, vitamin C also possesses prooxidant activity, which leads to H_2O_2-dependent cytotoxicity that significantly inhibited growth of cultured RCC cells and aggressive RCC xenografts in mice. The mechanism(s) of vitamin C production of H_2O_2 that induce preferential cell death in human RCC cells but not normal renal cells is unclear. One possible clue is that the lower expression of antioxidant enzymes, catalase, superoxide dismutase and glutathione peroxidase in tumor cells results in reactive oxygen species-induced tumorigenesis and sustained tumor progression [60]. However, although vitamin C-mediated RCC cytotoxicity can be abolished by exogenous catalase, the intracellular H_2O_2 production was decreased rather than increased over time following vitamin C exposure, as assessed by flow cytometric analysis using a probe of fichlorofluorescein diacetate (data not shown). Our data indicated that accumulation of intracellular H_2O_2 that causes DNA damage is not likely the mechanism by which vitamin C kills RCC cells. A recent study [61] demonstrated that vitamin C-mediated pancreatic cancer cell death was dependent on extracellular H_2O_2 formation with ascorbate radical as the electron donor. A second possible mechanism is that vitamin C generates extracellular H_2O_2 that targets membrane lipids, and forms hydroperoxides or reactive intermediates that are quenched or repaired in normal renal cells but not in sensitive RCC cells. New insights may follow from future studies of molecular profiling analysis of resistant and sensitive cells in regards to redox gene expression or signal transduction.

We have previously compared the *in vivo* activities between basal and vitamin D_3-induced Ad-hOC-E1 [29], and observed the lower degree of therapeutic efficacy by Ad-hOC-E1 in the absence of vitamin D_3. In the present study, we found that, although both Ad-hOC-E1/vitamin D_3 and vitamin C alone effectively slow down the progression of RCC tumors by 55% and 70%, respectively, complete tumor regression was observed by a combination of these three agents in three out of eight tumors (37.5%) treated. In addition, the remaining tumors receiving triple-agent therapy were also significant smaller than that treated with the other two protocols. It has been reported that, other than direct cytotoxicity towards cancer cells, vitamin C can affect cell migration and tube vessel formation of endothelial cells and thereby can inhibit angiogenesis [62]. The triple-pronged action of vitamin C in transcriptional activation of hOC promoter, apoptosis induction and anti-angiogenesis may account for the massive tumor regression in our experimental animal model of combination therapy.

In summary, the present study has provided the first demonstration that the human OC promoter was suitable for transcriptional targeting of RCC when combined with its transactivators vitamins C and D_3. Osteocalcin promoter-directed gene therapy using adenoviral vectors is undergoing clinical trials for targeting metastatic prostate cancer [24,26,63]. Both vitamin C [64–66] and vitamin D_3 [67,68] are also Food and Drug Administration-approved nutritional supplements used for cancer prevention and treatment. Our therapeutic strategy therefore could move rapidly from the preclinical development to the clinic by using agents that have been approved for clinical trials of tumors other than renal cancer and that may have benefits in RCC patients with poor prognosis and limited therapeutic options.

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Supporting information

Figure S1. Attenuation of vitamin D_3-induced OC expression in RCC cells by VDR-specific targeting siRNA. A representative RCC cell line (RCC42) transfected with VDR-specific targeting siRNA or nontargeting siRNA control (Ctr) was cultured in the presence or absence of 5 nM vitamin D_3 for 48 h and then subjected to RT-PCR analyses. MG63 treated with vitamin D_3 was used as the positive control for VDR and OC expression, and GAPDH was used as the RNA loading control.

Figure S2. Combination of hOC promoter-based adenoviral vectors and vitamins had no effect on the survival of normal renal epithelial cells *in vitro*. Normal renal epithelial cells (HRE) infected with (A) conditional replication-competent Ad-hOC-E1 and (B) Ad-hOC-TK at the indicated MOI were cultured in media containing either vitamin D_3 (5 nM) or vitamin C (0.15 mM), or both for 7 days. Additional 100ìg/ml ganciclovir (GCV) was used as the prodrug for Ad-hOC-TKinfected cells. Cytotoxicity was determined by crystal violent staining.

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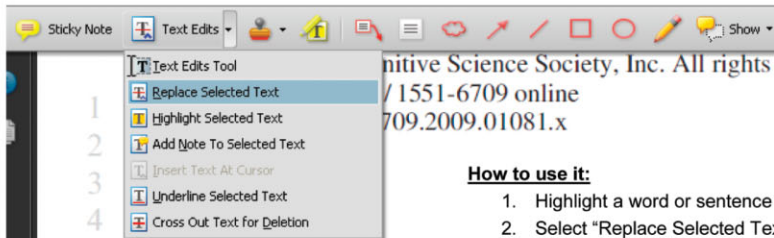

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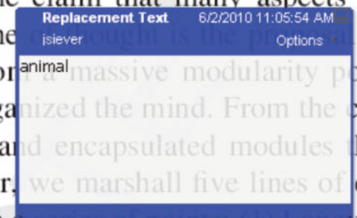

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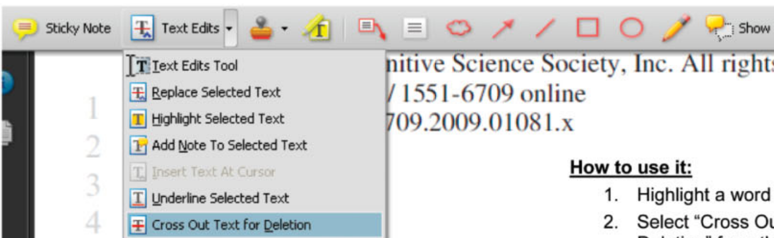

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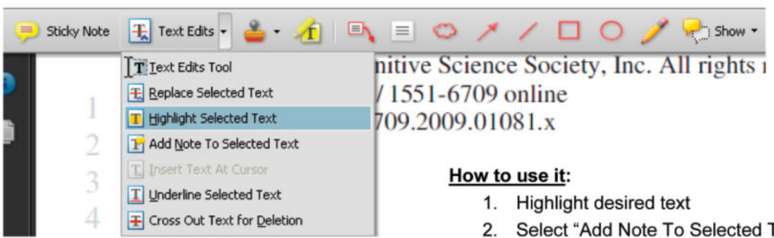

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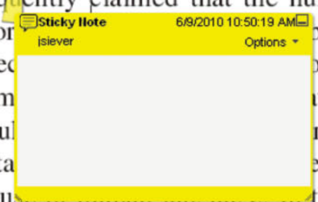

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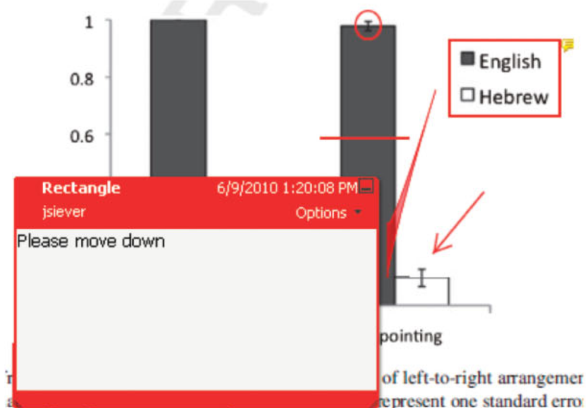

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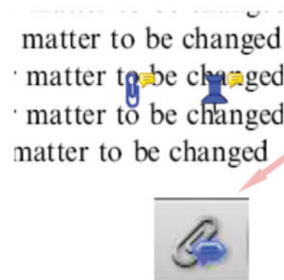

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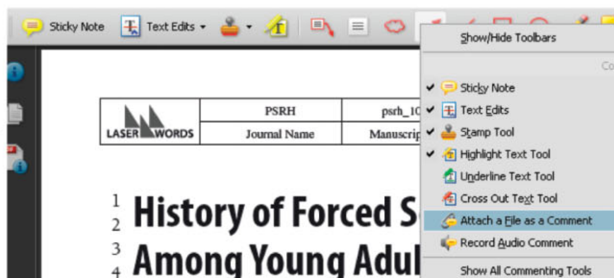

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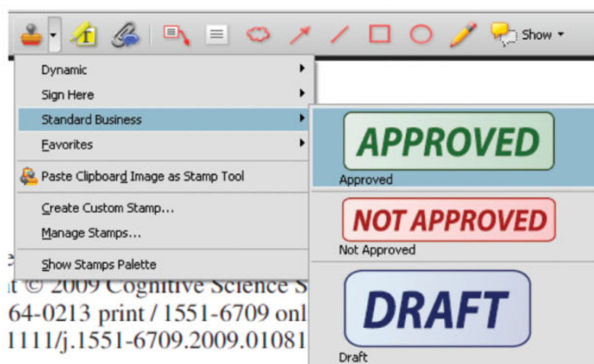

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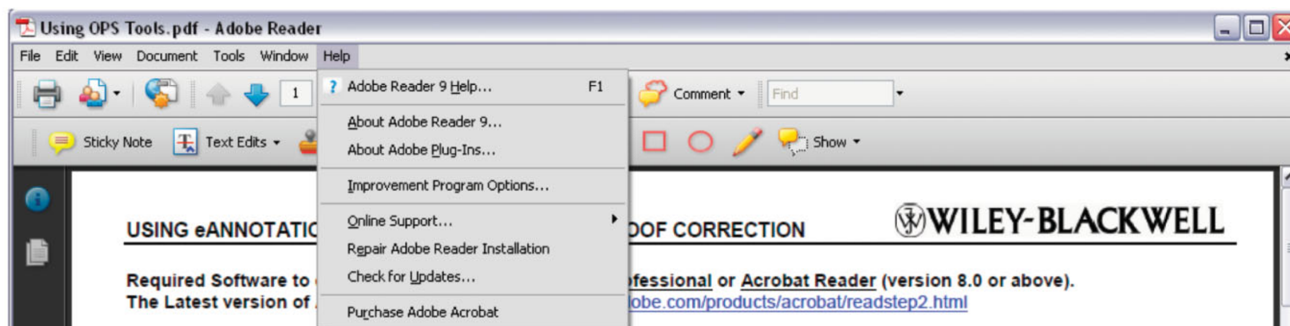

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