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Genistein induces the metastasis suppressor kangai-1 which mediates its anti-invasive effects in TRAMP cancer cells

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Abstract

Previous studies demonstrated a direct correlation with loss of kangai-1 (KAI1), a metastasis suppressor, and poor prognosis in human prostate and other cancers. In this study, we have characterized the age-dependent downregulation of KAI1 in the TRAMP model which was reversed when mice were fed a genistein-enriched diet. We demonstrated here that doses of genistein (5 and 10 μM)—achievable by supplement intake—significantly induced the expression of KAI1, both at the mRNA and protein levels (up to 2.5-fold), and decreased the invasiveness of TRAMP-C2 cells >2.0-fold. We have pinpointed KAI1 as the invasion suppressor, since its knockdown by siRNA restored the invasive potential of genistein-treated TRAMP-C2 cells to control levels. This work provides the first evidence that genistein treatment may counteract KAI1 downregulation, which is observed in many cancer types and therefore, could be used in anti-metastatic therapies.

Keywords: Genistein; KAI1/kangai; CD82; TRAMP; Prostate cancer; Metastasis; Phytoestrogen

It is estimated that more than 200,000 new cases of prostate cancer (CaP) will be diagnosed in the United States in 2007, of which >10% will die, making CaP the leading cause of cancer deaths in US men [1]. Although dysregulation of cell division is at the basis of tumor formation, the most serious threat to the patient's survival is the malignant cells' ability to invade surrounding tissues and form distant metastases [2]. The metastatic process is complex and involves several steps including invasion and angiogenesis [3]. Therefore, natural products which interfere with these processes are useful agents in the fight against cancer progression.

The reduced incidence of clinically relevant CaP in Asian males [4] suggests that the established chemopreventive action of the phytoestrogen, genistein, a major component of soy and a staple of the Asian diet, is not restricted to its anti-proliferative effects but might span invasion and metastasis. Previous studies have shown that genistein inhibits the invasion of different tumor cell lines. These effects were seen in prostate cancers among others [5]. Genistein has also been shown to reduce tumor metastasis in animal models [6]. Several mechanisms for these anti-metastatic properties have been proposed. Tumor invasion is a crucial part of the metastatic process and involves a number of steps including alterations in cellular adhesion and motility, proteolytic disruption of the basement membrane followed by migration through the extracellular matrix and the acquisition of an angiogenic phenotype [7]. Mechanistic studies have shown that genistein can hinder several of these steps. Genistein, at high doses has been shown to affect: cellular detachment by increasing the formation of focal adhesion complexes [8], invasion via downregulation of matrix metalloproteinases (MMPs): MMP-2, -9 [9], -8, -13 [10], as well as upregulation of tissue inhibitor of matrix metalloproteinases-1 (TIMP1) [9].

Kangai-1/CD82 (KAI1) was first identified as a prostate cancer metastasis suppressor [11]. Subsequent studies

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demonstrated a direct correlation with loss of KAI1 expression and poor prognosis in human prostate cancer among others [11]. A reduced KAI1 expression is associated with altered adhesion to extracellular matrix, and increased cell motility; which gives an increased invasive and metastatic potential to these cells.

Since genistein has been shown to reduce the invasive potential of several cell lines as well as metastasis *in vivo,* and given that the identification of genistein targets that mediate these effects remains crucial; we aimed at determining whether achievable genistein levels would modulate KAI1 expression in a prostate cancer cell line (TRAMP-C2) as well as in the Transgenic Adenocarcinoma Mouse Prostate model (TRAMP) and determine whether KAI1 contributes to genistein's anti-invasive effect.

Materials and methods

Cell culture and reagents. TRAMP-C2 cell line (gift from Dr. Norman M. Greenberg), was maintained at 37 °C with 5% CO_2 in phenol red-free IMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Quality Biologicals, Gaithersburg, MD), 2 mM glutamine, 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate (Sigma, St. Louis, MO). Twenty-four hours post-plating, genistein (Sigma) was added to a final concentration of 5 or 10 μM. Genistein containing media were replenished every day for the experiment duration. Control cells received equal amounts of ethyl alcohol, the solvent of genistein, in the media.

Animal handling and tissue preparation. TRAMP (The Jackson laboratory, Bar Harbor, Maine) and FVB mice (Charles River Laboratories, Wilmington, MA) were maintained at Georgetown University animal facilities. TRAMP mice were mated with FVB counterparts, and male offspring were genotyped as previously [12]. Four-weeks old transgenic males were fed genistein-free purified AIN-76A pellets (Harlan Teklad, Indianapolis, IN) supplemented with 0, 250, and 1000 mg genistein/kilogram diet ($n = 15$/diet group) (Sigma) until 20 weeks of age, for a total $n = 45$. Another group was kept on a regular diet and 10 mice were sacrificed at 5, 9, 18, and 24 weeks of age ($n = 10$/age group) for a total $n = 40$. Animal care and treatments were conducted in accordance with established guidelines and protocols approved by Georgetown University Animal Care and Use Committee. After completion of genistein treatment (20 weeks) or reaching endpoint ages, mice were sacrificed, blood collected, and all organs dissected out, weighed, fixed in 4% paraformaldehyde and paraffin-embedded. Portions of prostatic lobes (dorsolateral, ventral and anterior) were rapidly frozen on dry ice and stored at −80 °C, until processed for mRNA and protein analysis.

SiRNA treatment and plasmid transfection. For siRNA experiments, TRAMP-C2 cells were seeded at a density of 5×10^5 cells/6-well plate. After attachment, cells were treated with 0, 5 and 10 μM genistein for 4 days, counted and transfected with control or KAI1 siRNA using Transpass R1 siRNA transfection reagent (New England Biolabs, Ipswich, MA) at a final concentration of 100 nM. Twenty-four hours post-transfection, cells were treated with 0, 5 or 10 μM genistein for three more days and proteins were extracted or the invasion assay was performed after re-suspension. For plasmid transfection, TRAMP-C2 cells were transfected with pECFP-C1-Empty Vector or pECFP-C1-CD82 (Addgene plasmid 1818) [13] using GeneJammer transfection reagent (Stratagene, La Jolla, CA) for 3 days. Proteins were then extracted or the invasion assay performed.

Reverse transcription. Reverse transcription polymerase chain reaction (RT-PCR). RNA was extracted with TRIzol solution as suggested by manufacturer (Invitrogen). KAI1 or GAPDH genes were amplified using the Reverse-It one step kit (Abgene, Rochester, NY). Mouse specific primers were designed using Primer Quest program (IDT, Coralville, IA). KAI1-Forward 5′-TGAGGATTGGCCTGTGAACACTGA-3, KAI1-Reverse 5′-ATACTGGGAGCCAT T TCGAGCTGT-3′ and GAPDH-Forward 5′-GTGTTCCTACCCCCAATGTG-3′; GAPDH-Reverse: 5′-C TT GCTCA GTGTCCTTG CTG-3′. PCR reactions were as follows (94 °C-2 min → 94 °C-1 min (28×) → annealing temperature 58 °C-1 min → 72 °C-1 min → 72 °C-5 min. PCR products (440 and 349 base pairs, respectively) were separated on 1.5% agarose gels and visualized by ethidium bromide fluorescence using the Fuji LAS-1000 imager (Tokyo, Japan).

Western blot analysis. Protein extracts were prepared from TRAMP-C2 cells treated with or without 5 and 10 μM, siRNA transfected cells or plasmid transfected cells. Alternatively, proteins were extracted from dorsolateral prostates of TRAMP/FVB mice in the age or genistein treatment groups. Membranes were probed with anti-KAI1 antibody from Santa Cruz Biotechnologies (Santa Cruz, CA). Membranes were stripped and re-probed with GAPDH antibody from Abcam (Cambridge, MA) to ensure for equal loading. Molecular weight markers (Invitrogen) were run to confirm the size of immunoreactive proteins.

Immunofluorescence. TRAMP-C2 cells were plated on ECL-coated (Upstate, Charlottesville, VA) Lab–Tek chamber slides. After 7 days treatment with 0, 5 and 10 μM genistein, cells were fixed in methanol, blocked with 1% BSA at room temperature for 1 h then probed with KAI1 antibody (Santa Cruz) at a concentration of 1/50 overnight at 4 °C followed by incubation with Alexa Fluor secondary rabbit antibody (Molecular probes, Invitrogen) for 1 h. Slides were washed, counterstained with propidium iodide, mounted and viewed with a fluorescent Olympus BX 40 fluorescent microscope.

In vitro invasion assay. A quantitation of *in vitro* invasion of TRAMP-C2 cells was obtained from the Boyden Chamber assay (BD Biosciences) according to the manufacturer protocol. Briefly, after rehydration of chambers, 0.5 ml suspension of TRAMP-C2 (40,000 cells) in SFM (treated with or without genistein (5 and 10 μM) for 7 days, or 2) transfected with pECFP-C1-Empty Vector or pECFP-C1-CD82 for 3 days, or 3) treated with genistein (5 and 10 μM) for 4 days then with control siRNA or KAI1 siRNA for 3 days with or without genistein) was placed in the upper compartment of a BD Biocoat Matrigel Invasion Chamber with a 8 μm pore size polycarbonate filter coated with a thin Matrigel layer and incubated for 24 h at 37 °C with 10% FBS-supplemented media in the lower compartment. Non-migrating cells were removed with a cotton swab; remaining cells were fixed in methanol and stained with Toliudine Blue. Filters were removed from the chamber and mounted for visualization under the Olympus BX-40 microscope equipped with an Olympus DP-70 camera. Number of cells invading to the lower side of the filter was determined by counting the invaded cells in five random fields from triplicate filters for each treatment. Representative pictures were taken at a magnification of 10×.

Results

TRAMP cancer progression is associated with an age-dependent decrease in KAI1 levels which are retained by genistein consumption

To examine the effect of dietary genistein on KAI1 expression, we started by determining the levels of KAI1 transcripts and protein levels in the dorsolateral prostates (DLPs) of TRAMP/FVB mice of various ages (5, 9, 18, and 24 weeks of age). The histopathological grades of DLPs of TRAMP/FVB mice in the above-mentioned age groups were described previously [14] and represent normal, prostatic intraepithelial neoplasia (PIN), well-differentiated (WD) and poorly differentiated (PD) carcinoma, respectively. We have observed a statistically significant decrease (up to 50%) in KAI1 mRNA and protein levels by 18 weeks of age, followed by more than 90% reduction

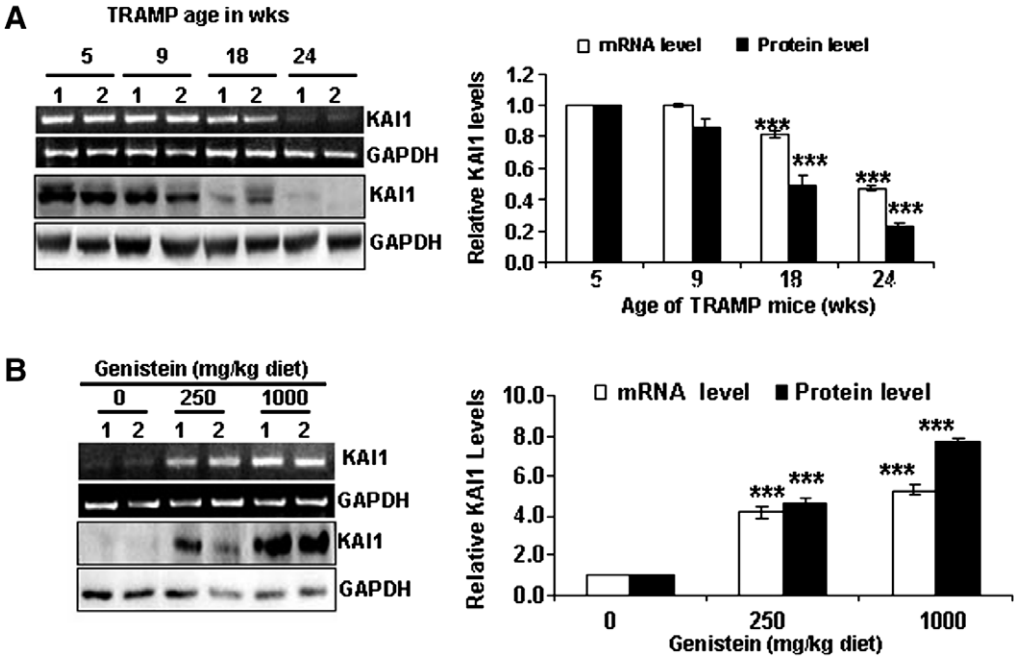

Fig. 1. Age-dependent KAI1 downregulation in dorsolateral prostates of TRAMP mice is reversed by dietary genistein. The relative mRNA and protein levels of KAI1 in age groups were determined by (1) RT-PCR: 500 ng RNA were subjected to 1-step RT-PCR with appropriate KAI1 and GAPDH primers. Photographs in (A) are representative of two samples (designated 1 and 2) from each age group. (2) WB: KAI1 protein levels were determined by running 50 μg proteins from DLP lysates on SDS–PAGE, and immunoblotting with KAI1 antibody. Immunoblots were stripped and probed for GAPDH to ensure equal loading. Values are the mean relative mRNA and protein levels ± SEM from three different blots, normalized to levels in normal DLPs. (B) Effect of genistein consumption on KAI1 levels in TRAMP DLPs was determined by (1) RT-PCR, similar to (A) with 500 ng of RNA from two samples of each genistein treatment group as well as from the control group. The quantitative analyses of PCR products are on the right. Values are the mean relative message levels ± SEM from three different agarose gels. (2) WB, similarly to (A), with lysates from two samples of each genistein treatment group as well as control group. The quantitative analyses of immunoblots are on the right. Values are the mean relative protein levels ± SEM from three different immunoblots, normalized to levels in normal DLPs. ***Indicates $p < 0.001$ in all parts.

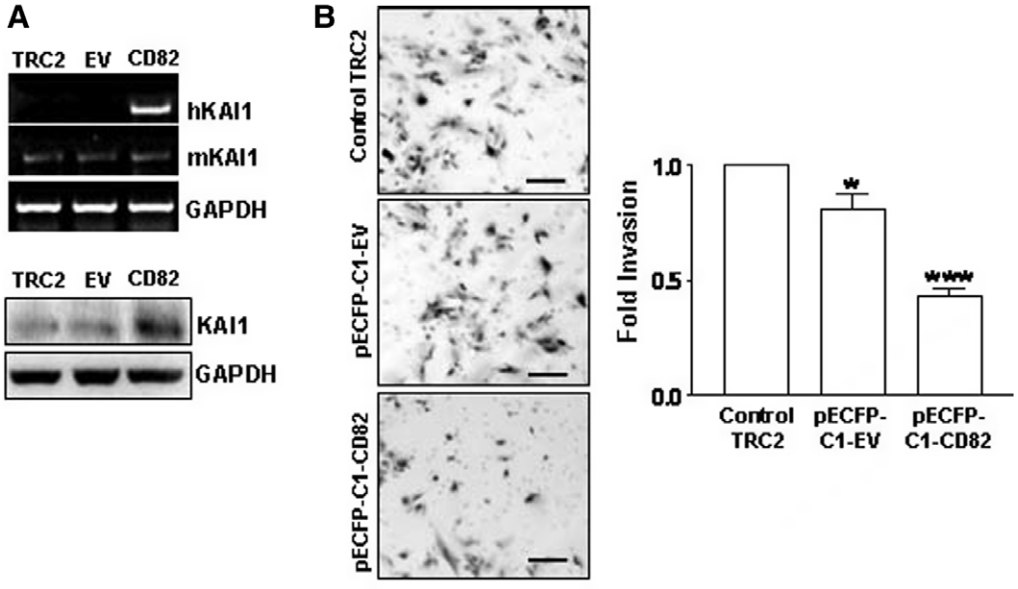

Fig. 2. KAI1 expression decreases invasion of TRAMP-C2 cells. Cells were untreated (TRAMP-C2 control), transfected with pECFP-C1-empty vector (EV) or pECFP-C1-CD82 (KAI1) for 3 days. (A) 500 ng of RNA from each treatment were subjected to 1 step-RT-PCR with primers for murine KAI1 (mKAI1), human KAI1 (hKAI1) and GAPDH (up). Equal amounts of protein lysates (50 μg) were analyzed by Western blotting using anti-KAI1 antibody or anti-GAPDH antibody (down). (B) Cells from above treatments were subjected to the BD biosciences Boyden Chamber assay (see Materials and methods). The invaded cells from three different filters for each treatment were counted and results were plotted as fold invasion normalized to number of invaded cells in the TRAMP-C2 control group. * and *** Indicate $p < 0.05$ and $p < 0.001$, respectively. Representative photographs are on the left, scale bar = 100 μm.

by 24 weeks of age (Fig. 1A). Concomitant with the reduction in the incidence of PD cancer in the DLPs of TRAMP/FVB mice consuming a genistein-rich diet that we previously reported [14], we observed a dose-dependent increase in KAI1 mRNA and protein levels (up to 4.0 and 7.0-fold) in the 250 and 1000 mg/kg diet groups, respectively (Fig. 1B).

KAI1 expression decreases the invasive ability of the TRAMP-derived cell line, TRAMP-C2

We next examined the levels of KAI1 message and protein in the TRAMP-derived TRAMP-C2 cell line. KAI1 (mRNA and protein) is expressed at very low levels in TRAMP-C2 cells (Fig. 2A). To determine the functional significance of KAI1 expression on TRAMP-C2 in terms of invasion, these cells were transiently transfected with a human KAI1 expression vector. The expression of hKAI1 was demonstrated via RT-PCR with human specific KAI1 primers (Fig. 2A). The successful expression of KAI1 protein was demonstrated by Western blot analysis (Fig. 2A, right). The transient expression of hKAI1 in TRAMP-C2 cells resulted in a statistically significant 2.0-fold decrease in the number of invading cells through a matrigel-coated 8 μm pore size polycarbonate filter after 24 h (Fig. 2B). This decrease was significantly different than the slight decrease observed upon transfection with the empty vector control, which we believe is due to the transfection reagent addition. Therefore, the reported 2.0-fold decrease in invasion is compared to empty vector-transfected cells.

Genistein treatment induces KAI1 expression and reduces the invasive potential of TRAMP-C2 cells in vitro

Having observed a significant dose-dependent increase in KAI1 protein levels in the DLPs of TRAMP/FVB mice

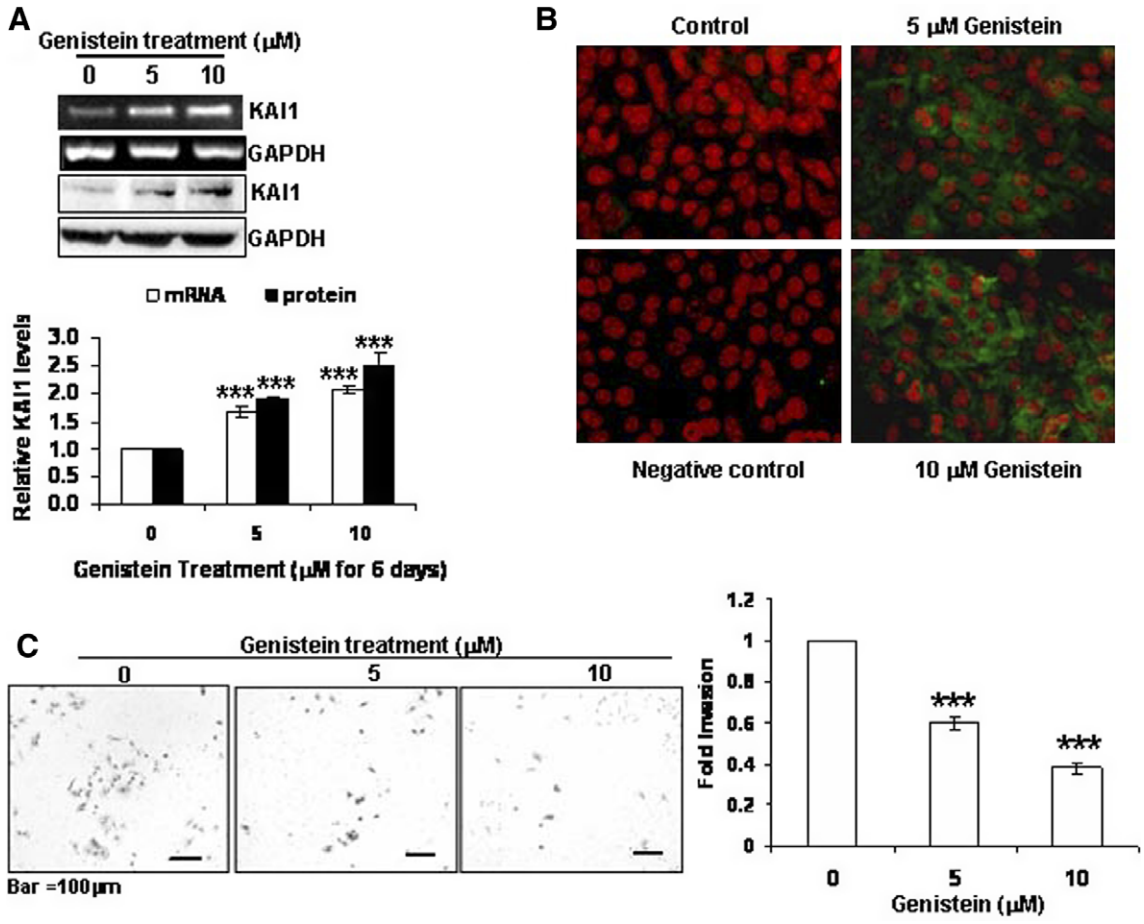

Fig. 3. Genistein induces the expression of KAI1 in TRAMP-C2 cells and reduces their invasive potential. Cells were treated with genistein (0, 5, and 10 μM) for 7 days. (A) KAI1 and GAPDH transcript and protein levels were analyzed by 1 step RT-PCR of 500 ng RNA from each treatment group and 50 μg protein lysates subjected to SDS–PAGE and immunoblotting with anti-KAI1 and anti-GAPDH antibodies. KAI1 mRNA and protein levels were quantified from three independent experiments and plotted with normalization to levels in untreated TRAMP-C2 cells (down). (B) KAI1 expression by immunocytochemistry with anti-KAI1 antibody. TRAMP-C2 were plated on chamber slides and treated with 0, 5 or 10 μM genistein for 7 days then fixed in methanol, incubated with Anti-KAI1 antibody (or antibody for negative control slide) and Alexa Fluor-tagged secondary antibody, counterstained with propidium iodide. Photographs were taken at 20× magnification (C) Cells from above treatments were subjected to the BD biosciences Boyden Chamber assay. The invaded cells from three different filters for each treatment were counted and results were plotted as fold invasion normalized to number of invaded cells in the untreated TRAMP-C2 control group. ***Indicates $p < 0.001$ in all parts. Representative photographs are on the left, scale bar = 100 μm.

fed the genistein-containing diets as compared to their age-matched control diet-fed counterparts, we aimed to determine whether genistein could induce the expression of KAI1 in TRAMP-C2 cells. Western blot analysis revealed that genistein treatment for 7 days resulted in a dose-dependent increase in KAI1 mRNA and protein levels (up to 2.5-fold in 10 μM treated cells) (Fig. 3A). This increase was also corroborated by increase in KAI1 immunoreactivity in TRAMP-C2 cells by immunofluorescence staining (Fig. 3B).

Previous studies have shown that genistein decreases the invasive potential of prostate cancer cells, albeit at supra-physiological levels (~50 μM). Therefore, we aimed at determining whether the low genistein doses used (5 and 10 μM) that induced the expression of KAI1, would affect TRAMP-C2 invasion. In fact, low doses of genistein treatment reduced the number of invaded cells by more than 60% in the 10 μM genistein group (Fig. 3C).

The invasiveness of genistein-treated TRAMP-C2 cells is restored by siRNA targeting KAI1

Having observed an induction of KAI1 levels by genistein concomitant with a decrease in invasion, we wanted to determine whether KAI1 increase plays a role in the observed invasion reduction. TRAMP-C2 cells were treated with 0 or 10 μM genistein (the dose that maximally induced KAI1) and subjected to control scrambled siRNA or KAI1 specific siRNA. The effectiveness of the siRNA has been demonstrated via Western blot analysis (Fig. 4A). These same cells were subjected to the Boyden chamber invasion assay and whereas 10 μM genistein of control siRNA-transfected cells decreased the number of invaded cells by more than 50% (Fig. 4B), this same dose failed to decrease the number of invaded cells transfected with KAI1 siRNA (Fig. 4B), suggesting that KAI1 expression is in fact responsible for the anti-invasive effects of genistein in our cell system.

Discussion

In this work, we have characterized for the first time the age-dependent decrease in KAI1 levels in the dorsolateral prostates of TRAMP/FVB mice and showed that KAI1 levels were restored in TRAMP/FVB mice consuming a genistein-rich diet, in a prevention regimen. The observed KAI1 retention in the DLPs of TRAMP/FVB mice does not seem to be solely due indirectly to the inhibition of PD cancer in TRAMP/FVB mice. In fact, we have shown that low levels of genistein can

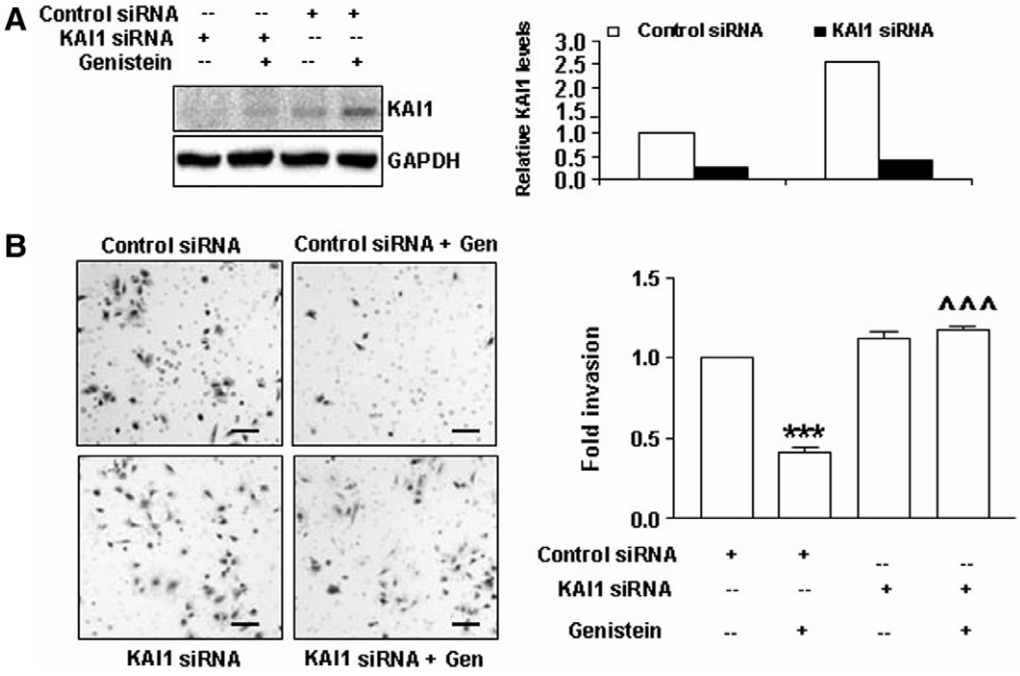

Fig. 4. KAI1 siRNA restores the invasive potential of genistein-treated TRAMP-C2 cells. (A) KAI1 protein levels in TRAMP-C2 cells transiently transfected with scrambled (control siRNA) or KAI1 siRNA for 72 h with or without 10 μM genistein after 4 days of genistein treatment (0 or 10 μM Quantitative analysis of KAI1 protein levels from duplicate experiments are shown on the right. (B) Evaluation of invasion after inhibition of KAI1 and genistein treatment (similarly to A). Cells from above treatments were subjected to the BD biosciences Boyden Chamber assay. The invaded cells from three different filters for each treatment were counted and results were plotted as fold invasion normalized to number of invaded cells in the untreated control siRNA transfected TRAMP-C2 control group. ***Indicates $p < 0.001$, compared to control siRNA transfected, untreated TRAMP-C2; ^^^ indicates $p < 0.001$ compared to control siRNA transfected, genistein-treated TRAMP-C2. Representative photographs are on the left. Scale bar = 100 μm.

induce KAI1 expression in the TRAMP-derived cell line, TRAMP-C2. This induction occurred at both the mRNA and protein levels, suggesting a transcriptional regulation of KAI1 by genistein.

The induction of KAI1 has been previously shown to be dependent on p53 [15]. However, we do not believe this to be the case in this study due to the SV40-transformed nature of the TRAMP model and its derived cell line. Other factors have been demonstrated to induce KAI1 re-expression including nerve growth factor and phorbol esters [16,17]. However, in each of these mentioned cases, it remains to be determined whether the effects on KAI1 expression are directly due to these agents or indirectly as a result of altered cell behavior. The common downregulation of KAI1 in metastatic disease does not involve loss of heterozygosity at the KAI1 locus or mutations within the KAI1 gene [11]. However, the KAI1 promoter is associated with a CpG island; and genistein has been shown to act as a demethylating agent *in vitro* at a similar dose as the one used in this work (5 μM) [18]; therefore, genistein might induce KAI1 expression via promoter demethylation, which remains to be determined.

The proximal region of the KAI1 promoter contains potential binding sites for several transcription factors including AP1 and Sp1 sites [19]. Phytoestrogens, such as genistein have been shown to regulate promoters containing such sites via estrogen receptor signaling [20]. Furthermore, a comprehensive cDNA microarray examining the effects of a similar dose of genistein (10 μM) revealed the induction of these AP-1 factors [21]. Therefore, KAI1 might be induced via AP-1 regulation by genistein.

We have also shown that the expression of KAI1 by transfection or genistein treatment reduced the invasive potential of TRAMP-C2 cells. The anti-invasive mechanism of KAI1 has not been fully elucidated. In fact, KAI1 lacks intrinsic activity, but mediates its functions via binding to surface receptors such as integrins or receptor tyrosine kinases in a cell specific manner [12]. Several attractive scenarios have been put forward to explain KAI1 anti-invasive effects; including upregulation of TIMP-1 [22] and the inactivation of urokinase receptor proteolytic activities [23]. Although previous studies have shown that several pro-and anti-metastatic factors are modulated by genistein treatment, these were done at supraphysiological levels and therefore might not recapitulate what is happening with the doses used in this study or what could be achieved via genistein consumption. In fact, through this work, we have shown that inhibition of KAI1 expression by siRNA restored the number of invaded cells to control levels in the presence of genistein. This result suggests that KAI1 induction is required for the decrease the invasiveness of TRAMP-C2 cells by genistein. This study suggests that genistein treatment could have beneficial effect in preventing metastatic disease by restoring KAI1 and abolishing the invasive potential of cancer cells.

Acknowledgments

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