Krüppel-Like Factor 4 Is a Novel Mediator of Selenium in Growth Inhibition

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Abstract
A previous prevention trial showed that selenium supplementation was effective in reducing (by 50%) the incidence of prostate cancer. Selenium has been reported to inhibit the growth of prostate cancer cells in vitro. Multiple mechanisms are likely to be operative in the underlying effect of selenium. Here, we report that Krüppel-like factor 4 (KLF4), a transcription factor of the KLF family, is an important target of selenium. We found that selenium up-regulates KLF4 expression and increases the DNA-binding activity of KLF4 in both the androgen-dependent LNCaP and the androgen-independent PC-3 human prostate cancer cells. The increase of KLF4 mRNA is accounted for primarily by enhanced transcription, although the contribution of a slight abatement in mRNA degradation cannot be ruled out. KLF4 knockdown using short interference RNA significantly weakens the effects of selenium on DNA synthesis inhibition, apoptosis induction, and the expression of three KLF4 target genes, cyclin D1, p21/WAF1, and p27/Kip1. In addition, the overexpression of KLF4 not only leads to an induction of apoptosis in the control cells, but also enhances the DNA synthesis-suppressive and -proapoptotic activities of selenium. Taken together, our results suggest that KLF4 plays a key role in mediating the growth-inhibitory effect of selenium in prostate cancer cells.

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Introduction
KLF4, also known as gut-enriched Krüppel-like factor (GKLF) or epithelial zinc finger, is a member of the Krüppel-like (KLF) family of proteins (1). KLF proteins are known to play an important role in differentiation and development (2, 3). They are zinc finger transcription factors, characterized by a COOH-terminal DNA-binding domain which consists of three C2H2 zinc fingers (2, 3). KLF4 is highly expressed in terminally differentiated epithelial cells of the skin and gastrointestinal tract (4, 5). It regulates the expression of a number of genes essential for cell cycle progression (e.g., cyclin D1, cyclin B1, p21/WAF1, p27/Kip1, inhibitor of DNA-binding 3, ornithine decarboxylase 1), and is intimately involved in controlling G1-S and G2-M checkpoints following DNA damage (6-13). The level of KLF4 generally increases in response to serum deprivation (13), contact inhibition (1), and heat stress (14).

Depending on cell type or cell context, KLF4 may act either as a tumor suppressor gene or as an oncogene (15, 16). The function of KLF4 in carcinogenesis has been studied more extensively in gastrointestinal cancer than in other cancers. KLF4 is down-regulated in colon (17, 18), gastric (19), and esophageal (20-22) cancers. The reduction is due to either a loss of heterozygosity of the KLF4 locus, hypermethylation of the 5′-untranslated region, or point mutations in the coding region (18). Overexpression of KLF4 is known to decrease the tumorigenicity of colon cancer cells (23). On the other hand, KLF4 knockout mice have been reported to manifest differentiation defects and precancerous changes in the stomach (24). Consistent with its tumor suppressor activity in gastrointestinal tumors, decreased KLF4 expression has also been observed in other types of cancer, including prostate (25, 26), bladder (27), lung (28), and T-cell leukemia (29). Conversely, the role of KLF4 as an oncogene is supported by the finding of an increased expression in oral dysplastic epithelium, squamous cell carcinoma (30), and breast cancer (25) when compared with the corresponding normal tissues. Given that the deregulation of KLF4 is a common occurrence in organ site carcinogenesis, identifying a corrective strategy and studying its consequence on cancer cell growth would be an endeavor worthy of pursuit.

Prostate cancer is characterized by a long latency period and is therefore most suited for testing the idea of intervention. In a previous randomized, placebo-controlled cancer prevention trial, supplementation with a nutritional dose of selenium was found to reduce prostate cancer incidence by 50% (31, 32). In vitro studies showed that selenium inhibits human prostate cancer cell growth, blocks cell cycle progression, and induces programmed cell death (33, 34). In an effort to delineate the molecular targets underlying the anticancer action of selenium, we did a microarray analysis to profile gene expression changes
mediated by selenium in the PC-3 human prostate cancer cells (33). KLF4 was identified as one of the early selenium-responsive genes. A rapid induction of KLF4, accompanied by the altered expression of several known KLF4 targets (e.g., cyclin D1, p21/WAF1, p27/Kip1), were observed in response to selenium treatment. Based on the above information, it would be reasonable to hypothesize that KLF4 is an important proximal mediator of the action of selenium. In this report, we followed up on our microarray data by examining the up-regulation of KLF4 and the modulation of KLF4 targets by selenium. Additionally, we evaluated how KLF4 knockdown or overexpression might modify the antiproliferative and proapoptotic activities of selenium. The selenium compound used in the study is methylseleninic acid (CH₃SeO₂H, abbreviated to MSA), which was developed specifically for in vitro experiments (35). Once taken up by cells, MSA is rapidly reduced to the active metabolite, methylselenol (which is rather unstable in itself), via a nonenzymatic reaction involving glutathione and NADPH.

**Results**

**MSA Up-Regulates KLF4 mRNA Expression**

We assessed KLF4 mRNA level by quantitative real-time reverse transcription-PCR (RT-PCR) in PC-3 and LNCaP human prostate cancer cells. Cells were treated with 10 μmol/L of MSA for various lengths of time as indicated in Fig. 1. In both cell lines, a 2-fold induction of KLF4 mRNA was detected as early as 2 h after exposure to MSA. In LNCaP cells, the increase plateaued off at this point, whereas in PC-3 cells, it continued to increase to ~3.5-fold at 3 h, and remained at this level for at least up to 16 h. We next set out to examine the level of KLF4 protein in response to MSA treatment. Unfortunately, none of the commercially available KLF4 antibodies produced a specific signal on Western blots.

**MSA Increases KLF4 mRNA Stability and Transcription Initiation**

To determine whether the up-regulation of KLF4 mRNA was a result of decreased mRNA degradation or increased transcription, we carried out an mRNA stability assay under conditions in which new RNA synthesis was blocked. Actinomycin D was added to the culture at the time of MSA treatment, and KLF4 mRNA levels were followed in a 6-h time course experiment (Fig. 2). Our results showed that MSA treatment modestly increased the stability of KLF4 mRNA in PC-3 cells. The shift in the decay curve was less notable in LNCaP cells.

We then studied the effect of MSA on KLF4 transcription by nuclear run-on assay. Biotin-labeled nascent transcripts obtained by run-on transcription were isolated by using streptavidin particle beads, and quantitated by real-time RT-PCR. As shown in Fig. 3, MSA treatment resulted in a 2- to 3-fold induction of KLF4 transcription at the 3- and 6-h time points in both cell lines. The data thus indicate that the elevation of KLF4 mRNA level by MSA is primarily due to an

![FIGURE 1](image1)

**FIGURE 1.** Effect of MSA on KLF4 mRNA expression as determined by real-time RT-PCR. Columns, mean percentage of control; bars, SE. With the exception of the 1-h data point in LNCaP cells, the remaining data points are statistically different (P < 0.01) from the untreated control.

![FIGURE 2](image2)

**FIGURE 2.** Effect of MSA on KLF4 mRNA stability. Points, mean percentage of control; bars, SE.
increase in transcription initiation. The contribution from increased mRNA stability seems to be relatively minor.

Gene transcription is generally controlled by promoter regions. Because major regulatory sequences are usually located near the basal transcriptional machinery, we analyzed the effect of MSA on the activity of a KLF4 proximal promoter. Cells were transiently transfected with a luciferase reporter gene construct containing 1 kb of the proximal promoter region and 550 bp of the 5’-untranslated region of the KLF4 gene. The reporter gene assay was done during a 16-h time course. MSA treatment did not lead to any significant change in the activity of this proximal KLF4 promoter (data not shown). We are in the process of conducting further upstream cloning to identify the region responsible for MSA up-regulation of KLF4.

Knock-Down of KLF4 Attenuates the Effect of MSA on the Modulation of KLF4 Target Genes

Our previous microarray analysis of PC-3 cells identified three KLF4 target genes which were modulated by MSA (33). These genes are cyclin D1 (0.6), p21/WAF1 (3.5), and p27/Kip1 (2). The number in parentheses denotes the treatment to control ratio. A value of <1 or >1 signifies a decrease or an increase, respectively. To delineate the role of KLF4 as a mediator of these MSA-modulatable genes, we transiently transfected PC-3 cells with a Stealth KLF4 short interference RNA (siRNA) duplex to knock down KLF4 expression. As shown in Fig. 5A, the siRNA markedly inhibited KLF4 mRNA expression, not only in the basal condition, but also when cells were treated with MSA. The response of the KLF4 target genes to MSA was determined by real-time RT-PCR in the KLF4 knockdown cells or the scrambled control siRNA-transfected cells. MSA treatment resulted in a down-regulation of cyclin D1, and an up-regulation of p21/WAF1 and p27/Kip1 in the scrambled control-transfected cells (Fig. 5B, C and D). KLF4 knockdown significantly muted the effect of MSA on the expression of these genes.

KLF4 Up-Regulation Contributes to MSA-Mediated Growth Inhibition

In an effort to evaluate the biological significance of KLF4 up-regulation by MSA, we assessed the response of the KLF4 knockdown cells to MSA-mediated DNA synthesis inhibition and apoptosis induction. BrdU ELISA and Cell Death ELISA were conducted at 16 h post–MSA treatment. In the absence of MSA, DNA synthesis and apoptosis were not altered by KLF4 knockdown (data not shown), probably due to the relatively low basal level of KLF4 in the cells. MSA treatment inhibited DNA synthesis by >85% in the scrambled control-transfected cells, as opposed to 65% in the KLF4 knockdown cells (Fig. 6A). The difference is statistically significant (P < 0.01). Additionally, as presented in Fig. 6B, MSA-induced apoptosis was markedly muted as a result of KLF4 knockdown.

As a flip side to the knockdown experiment, we transiently transfected PC-3 cells with a KLF4 expression vector, and assessed the response of KLF4-overexpressing cells to MSA by BrdU ELISA and Cell Death ELISA. With the treatment of 5 μmol/L of MSA for 16 h, DNA synthesis was suppressed by 37% and 50% in the mock-transfectants and the KLF4-transfectants, respectively (Fig. 7A). The difference is statistically significant (P < 0.05). In the absence of MSA, KLF4 overexpression did not affect DNA synthesis (data not shown), but significantly induced apoptosis (Fig. 7B, first two columns). The effect of MSA on apoptosis was enhanced by ~60% as a result of KLF4 overexpression (Fig. 7B, last two columns). We
lowered the dose of MSA from 10 to 5 μmol/L for the KLF-transfection experiment in order to leave room to detect an enhanced effect of MSA by KLF4 overexpression. When taken together with the knockdown data, our results showed the important role of KLF4 up-regulation in mediating the effect of MSA on growth inhibition and apoptosis.

Discussion

In this report, we present three lines of evidence to support the role of KLF4 in mediating the effect of selenium on growth inhibition and apoptosis induction in prostate cancer cells. First, selenium treatment leads to a rapid induction of KLF4 expression and DNA-binding activity. Second, KLF4 knockdown by siRNA significantly diminishes the responsiveness to selenium with respect to DNA synthesis inhibition, apoptosis induction, and the expression of three KLF4 target genes, cyclin D1, p21/WAF1, and p27/Kip1. Third, overexpression of KLF4 enhances the DNA synthesis suppressive and proapoptotic effects of selenium. It is important to put into perspective that KLF4 is one of several transcription factors of which the expression is known to be modulated by selenium at early time points of treatment. Included in this group of transcription factors are GADD153, androgen receptor, FOXO, FOXA1, ATF6, XBP1, and nuclear factor-κB (33, 36-43). Although each is reputed to regulate a different spectrum of downstream targets, a common denominator among the targets is their involvement in controlling cell proliferation and apoptosis. Thus, it is not surprising to find that knocking down KLF4 alone may not completely block the effect of selenium on growth suppression.

Other members of the KLF family may also contribute to the action of selenium. To date, 21 proteins in the KLF family have been identified in humans (44). They share a highly conserved carboxyl-terminal zinc finger DNA-binding domain and a similar DNA-binding consensus element (44). Some of these proteins contain identical DNA-interacting amino acids, and therefore, bind to the same DNA element (44). Our EMSA study with a KLF4-consensus element showed an increase in the formation of three specific DNA-protein complexes as a result of selenium treatment, whereas only one of the complexes could be supershifted by the KLF4 antibody. Because Sp1 has been shown to compete with KLF4 for DNA binding (44), we conducted supershift assays with a Sp1 antibody. However, none of the complexes were affected by the addition of the Sp1 antibody (data not shown). Our microarray data set shows that KLF5 is also increased 2- to 3-fold by selenium (33). KLF5 has been reported to be frequently down-regulated and functions as a potential tumor suppressor in prostate cancer (45). Hence, the possible involvement of KLF5 in the action of selenium deserves further investigation.

In the present study, we showed that selenium down-regulates the expression of cyclin D1, and up-regulates the expression of two CDK inhibitors, p21/WAF1 and p27/Kip1. The combined effect of selenium on these key cell cycle regulators is consistent with its activity in blocking cell cycle progression. Knocking down KLF4 in selenium-treated cells restores the expression of these genes to their control levels. In contrast, KLF4 knockdown in the untreated cells fails to bring about any changes. It is possible that in the basal condition, the transcription of these genes in prostate cells is under the control of a number of transcription factors. Knocking down one particular transcription factor would not be sufficient to significantly affect their expression. With selenium treatment, KLF4 may assume a more dominant role in the regulation of these genes.

The role of KLF4 in growth regulation is cell type– and cell context–specific (15, 16). It could either inhibit or promote cell

FIGURE 4. Effect of MSA on KLF4 DNA-binding activity in PC-3 and LNCaP cells. EMSA (A and B) and supershift assay (C) with a KLF4-consensus element. A. Increase of KLF4 DNA-binding activity by MSA in PC-3 cells. B. Increase of KLF4 DNA-binding activity by MSA in LNCaP cells. s, specific competitor (wild-type KLF4-consensus element); n, nonspecific competitor (mutant KLF4-consensus element); K, anti-KLF4 antibody; E, anti-Ets1 antibody.
growth (15, 16). Here, we show that transfection of KLF4 in prostate cancer cells leads to a significant induction of apoptosis. Together with the observation of decreased KLF4 expression in prostate cancer (25, 26), the data are supportive of a potential tumor-suppressing function of KLF4 in prostate cancer. Our study also suggests a proapoptotic activity of KLF4. However, none of the known targets of KLF4 have been implicated as key regulators of apoptosis. It is imperative to identify novel targets of KLF4 in order to better understand the mechanism underlying the proapoptotic function of KLF4 in prostate cancer cells.

Materials and Methods

Selenium Reagent and Prostate Cell Culture

MSA was synthesized as described previously (35). The PC-3 and LNCaP human prostate cancer cells were obtained from American Type Culture Collection. The cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum.

FIGURE 5. Effect of KLF4 siRNA knockdown on KLF4 target gene expression in PC-3 cells as detected by real-time RT-PCR. Columns, mean; bars, SE (n = 3). *, P < 0.01, statistically different from the untreated scrambled control.

FIGURE 6. A. Effect of KLF4 siRNA knockdown on MSA inhibition of DNA synthesis as detected by ELISA of BrdUrd incorporation. Columns, mean; bars, SE (n = 3). B. Effect of KLF4 siRNA knockdown on MSA induction of apoptosis as detected by ELISA of DNA fragmentation. The results are expressed as an increase of apoptosis compared with the untreated control. *, P < 0.01, statistically different from scrambled siRNA-transfected samples.
serum, 2 mmol/L of glutamine, 100 units/mL of penicillin, and 100 µg/mL of streptomycin.

**Nuclear Lysate Preparation**

Nuclear protein extracts were prepared as described previously (36). Cells were harvested, washed twice with PBS, and resuspended in a hypotonic buffer [10 mmol/L HEPES-KOH (pH 7.9), 1.5 mmol/L MgCl₂, 10 mmol/L KCl, and 0.1% NP40] and incubated on ice for 10 min. Nuclei were precipitated with 3,000 × g centrifugation at 4°C for 10 min. After washing once with the hypotonic buffer, the nuclei were lysed in a lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, and 1% Triton X-100] and incubated on ice for 30 min. The nuclear lysate was precleared by 20,000 × g centrifugation at 4°C for 15 min. Protein concentration was determined by using the bicinchoninic acid protein assay kit (Pierce).

**EMSA**

EMSA was conducted as previously described with 10 µg of nuclear protein extract and 1 ng of end-labeled double-stranded oligonucleotide probe (46). The unlabeled oligonucleotide competitors were present at 100× in excess in the competition experiments. The sequences of the oligonucleotides used in the assays are as follows: the wild-type KLF4-consensus element, 5′-ATGCAGGAGAAAGAAGGGCGTAGTATCTACTAG-3′ (47); the mutated KLF4-consensus element, 5′-ATGCAGGAGAAAGAAGTTCGTATCTACTAG-3′ (47). For the super-shift assays, the antibody against KLF4 or Ets-1 (both from Santa Cruz Biotechnology) was added after the formation of the protein-DNA complexes, and incubation was continued for an additional 30 min on ice. The reaction mixtures were analyzed by 5% PAGE, and the gel was subsequently dried and visualized by autoradiography.

**Real-time RT-PCR**

The analysis was done as described previously (36) by using RNA isolated with the TRIzol reagent (Invitrogen). The PCR primers and TaqMan probes for β-actin (a housekeeping gene, assay ID Hs99999903_m1), KLF4 (assay ID Hs00358836_m1), cyclin D1 (assay ID Hs00277039_m1), p21/WAF1 (assay ID Hs00355782_m1), and p27/Kip1 (assay ID Hs153227_m1) were Assays-on-Demand products from Applied Biosystems. The PCR conditions were as follows: an initial incubation at 50°C for 2 min, then adenaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Temperature cycling and real-time fluorescence measurement were done by using an ABI Prism 7900 Sequence Detection System (Applied Biosystems). The relative quantitation of gene expression was done by using the comparative Ct (ΔΔCt) method (48). Each real-time RT-PCR experiment was done in triplicate, and the mean Ct value was used for data analysis. The final result is presented as the mean of three separate experiments ± SE.

**KLF4 mRNA Stability Assay**

Actinomycin D (5 µg/mL) was added to the cultures to stop new RNA synthesis at the time of MSA treatment, and KLF4 mRNA levels were measured by real-time RT-PCR at the 2, 4, and 6 h time points. The turnover of KLF4 mRNA was determined by comparing mRNA levels over time in cells treated with or without MSA.

**Reporter Gene Assay**

The KLF4-pGL2-luciferase reporter gene construct (49), containing 1 kb of the 5′-flanking region and 550 bp of the 5′-untranslated region of the KLF4 gene, was kindly provided by Dr. Vincent W. Yang at Emory University. This plasmid was transiently transfected into cells with the LipofectAMINE and Plus reagents (Invitrogen) at a concentration of 4 µg per 10 cm culture dish. After incubating with the transfection mixture for 3 h, the cells were trypsinized and replated in triplicate onto six-well plates. Cells were allowed to recover for an additional 24 h before exposure to 10 µmol/L of MSA. After 1, 2, 3, 6, or 16 h of MSA treatment, cells were lysed in reporter lysis buffer (Promega), and the luciferase activity was assayed by using the Luciferase Assay System (Promega). Protein concentration in cell extracts was determined with the bicinchoninic acid protein assay kit.
assay kit (Pierce). Luciferase activities were normalized by the protein concentration of the sample. The transfection experiments were repeated thrice.

Quantitative Nuclear Run-on Assay
Run-on transcription was done according to a previously described method with biotin-16-UTP (Roche; ref. 50). Biotin-labeled nascent transcripts were then isolated by using streptavidin particle beads, and quantitated by real-time RT-PCR. The experiment was done thrice in triplicate.

Transient Transfection of a KLF4 Expression Vector
The KLF4 expression vector, pcDNA3.1/His B-GKLF (51), was a gift from Dr. Anil K. Rustgi at the University of Pennsylvania. The transfection was carried out by using the LipofectAMINE and Plus reagents (Invitrogen) according to the instructions of the manufacturer. At 24 h before transfection, cells were plated in growth medium without antibiotics at a density to reach 90% to 95% confluency at transfection. The KLF4 expression vector or the pcDNA3.1/His B mock plasmid was introduced into cells at a concentration of 4 µg per 1 cm culture dish. The DNA/liposome mixture was removed at 3 h after transfection. The cells were trypsinized 16 h later and replated in triplicate onto two 96-well plates, one for BrdU ELISA and the other for Cell Death ELISA. Cells were allowed to recover for an additional 24 h before exposure to 5 µmol/L of MSA. BrdU ELISA and Cell Death ELISA were conducted 16 h post–MSA treatment.

RNA Interference
A set of three predesigned Stealth Select siRNA duplexes to the human KLF4 gene as well as the Stealth siRNA Negative Control Duplex with medium GC content were purchased from Invitrogen. The KLF4 siRNA duplex with the highest efficiency in knocking down KLF4 expression was used for the experiment. The sequence of this siRNA (KLF4-HSS113796) was 5'-GGACCCUGGACUUUAUCUCUCCA-U-3', corresponding to nucleotides 611 to 635 of the human KLF4 cDNA sequence (GenBank accession no. NM_004235).

The siRNAs were introduced into cells by using the LipofectAMINE 2000 reagent (Invitrogen). Transfection efficiency and optimal transfection condition were determined by using the BLOCK-iT fluorescent RNA duplex (Invitrogen), which is not homologous to any known gene. At 24 h prior to transfection, cells were plated in triplicate in growth medium without antibiotics at a density to reach 60% to 70% confluency at transfection. The siRNAs were transfected into cells at a concentration of 80 nmol/L. The cells were treated with 10 µmol/L of MSA at 24 h after transfection. Real-time RT-PCR analysis, BrdU ELISA, and Cell Death ELISA were conducted at 16 h post–MSA treatment.

Cell Proliferation Assay
Proliferation was measured by using the BrdU Cell Proliferation ELISA kit (Roche) according to the instructions of the manufacturer with minor modifications. Briefly, after labeling the cells with bromodeoxyuridine (BrdUrd) for 2 h, the WST-1 reagent (Roche), which quantitatively monitors the metabolic activity of the cells, was added to the wells to a final concentration of 10%. The cells were incubated for an additional 2 h. The amount of formazan converted from WST-1 by the metabolically active cells was quantitated at 450 nm. After removing the medium, the cells were fixed and the DNA denatured for the incorporated BrdUrd to bind to a peroxidase-conjugated anti-BrdUrd antibody. The immune complexes were detected by the subsequent substrate reaction, and the reaction product was quantified by absorbance at 370 nm (reference wavelength at 492 nm). Culture medium without cells and cells incubated with the anti-BrdUrd peroxidase antibody in the absence of BrdUrd were used as controls for nonspecific binding. The BrdU ELISA result was normalized by the WST-1 reading, which correlates directly with the cell number. The experiment was done thrice in triplicate.

Apoptosis Detection
Detached cells were precipitated by centrifugation and pooled with attached cells. Cytoplasmic histone-associated DNA fragments were quantified by using the Cell Death Detection ELISA PLUS kit (Roche Applied Science) according to the protocols of the manufacturer. The absorbance was measured at 405 nm (reference wavelength at 492 nm). The experiment was done thrice in triplicate.

Statistical Analysis
Mean activities were calculated from three independent experiments done in triplicate. The Student’s two-tailed t test was used to determine significant differences between treatment and control values.

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