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Silencing KRAS Overexpression in Cadmium-Transformed Prostate Epithelial Cells Mitigates Malignant Phenotype

Ntube N.O. Ngalame, Michael P. Waalkes, and Erik J. Tokar*

Stem Cell Toxicology Group, National Toxicology Program Laboratory, Division of the National Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709, United States

Abstract

Cadmium (Cd) is a potential human prostate carcinogen. Chronic Cd exposure malignantly transforms RWPE-1 human prostate epithelial cells into CTPE cells by an unclear mechanism. Previous studies show that RWPE-1 can also be malignantly transformed by arsenic, and KRAS activation is key to causation and maintenance of this phenotype. Although Cd and arsenic can both transform prostate epithelial cells, it is uncertain whether their mechanisms are similar. Thus, here we determined whether KRAS activation is critical in causing and maintaining Cd-induced malignant transformation in CTPE cells. Expression of KRAS, miRNAs, and other genes of interest was analyzed by Western blot and RT-PCR. Following stable KRAS knockdown (KD) by RNA interference using shRNAmir, the malignant phenotype was assessed by various physical and genetic parameters. CTPE cells greatly overexpressed KRAS by 20-fold, indicating a likely role in Cd transformation. Thus, we attempted to reverse the malignant phenotype via KRAS KD. Two weeks after shRNAmir transduction, KRAS protein was undetectable in CTPE KD cells, confirming stable KD. KRAS KD reduced stimulated RAS/ERK and PI3K/AKT signaling pathways and markedly mitigated multiple physical and molecular malignant cell characteristics including: hypersecretion of MMP-2, colony formation, cell survival, and expression of cancer-relevant genes (reduced proliferation and cell cycle-related genes; activated tumor suppressor *PTEN*). However, KRAS KD did not reverse miRNA expression originally down-regulated by Cd transformation. These data strongly suggest KRAS is a key gene in development and maintenance of the Cd-induced malignant phenotype, at least in the prostate. It is not, however, the only genetic factor sustaining this phenotype.

Graphical Abstract

*Corresponding Author: tokare@mail.nih.gov. Tel: 919-541-2328. Fax: 919-541-3970.

Notes

The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemres-tox.6b00137. Genes and primers analyzed by real time RT-PCR; effects of KRAS knockdown on selected dysregulated miRNAs (PDF)

INTRODUCTION

Cadmium (Cd) and Cd compounds are toxic environmental pollutants. A known human carcinogen, Cd is implicated in prostate cancer with substantial evidence in both humans and animals.^{1,2} Human exposure to Cd can be occupational, or through food consumption, tobacco use, and inhalation of ambient air.^{2,3}

Cd is able to malignantly transform normal human prostate epithelial cells (RWPE-1) *in vitro*, and the transformed cells (designated Cd transformed prostate epithelial or CTPE) rapidly form highly invasive adenocarcinomas after inoculation in xenograft studies with mice.⁴ This *in vitro* work⁴ provides supportive evidence that Cd is a human prostatic carcinogen acting directly at the level of the epithelial cells, and it provides a model with human relevance to help elucidate mechanisms of Cd-induced carcinogenesis, which are incompletely defined.

Cd shares several similar characteristics with another human inorganic carcinogen, arsenic^{5,6} such as common carcinogenic targets, including potentially the prostate,² and the potential to assist in local spread of malignancies by recruiting nearby normal stem cells into a cancer stem cell phenotype.^{7,8} However, it is unknown whether Cd and arsenic share similar carcinogenic mechanisms. For example, both metals can transform the same normal human prostate epithelial cell line (RWPE-1) *in vitro* into a cancer phenotype.^{4,5} However, Cd requires much less time than inorganic arsenic (8 versus 29 weeks, respectively) in order to transform the RWPE-1 cells, suggesting that the mechanisms of Cd carcinogenesis likely differ from those of the metalloid arsenic.

KRAS (Kirsten Rat Sarcoma Viral Oncogene Homologue) is a small GTP-binding protein that is key to controlling many cellular processes, including proliferation, differentiation, and survival.⁹ KRAS activation is common in cancers, including prostate cancer.^{9,10} Previous studies indicate that KRAS activation is key in the malignant transformation and maintenance of malignant phenotype of arsenic-transformed human prostate epithelial (CAsE-PE) and prostate stem cells (As-CSCs).^{11–13} Indeed, silencing KRAS overexpression in these transformants partially mitigates their cancer phenotype through the loss of multiple physical and molecular cancer cell characteristics.^{12,13}

Although KRAS activation can be an important event in prostate carcinogenesis,¹⁴ it has not been shown to be activated in Cd-transformed prostate epithelial CTPE cells. Thus, in this

study, we examined whether KRAS activation also occurs with Cd transformation of these prostate cells. Based on initial findings, we also determined the role of KRAS in causing and maintaining the cancer phenotype by silencing the KRAS expression. The findings in CTPE cells (Cd transformant) were compared to those previously shown in the isogenic CAsE-PE cells (arsenic-transformant) to help determine if the two inorganics share similar mechanisms of carcinogenesis.

MATERIALS AND METHODS

Chemicals and Reagents

Keratinocyte-serum free medium (K-SFM), bovine pituitary extract (BPE), epidermal growth factor (EGF), and 100X antibiotic-antimycotic mixture were purchased from Life Technologies, Inc. (Grand Island, NY). GIPZ lentiviral KRAS shRNAir particles (catalog no. VGH5523, clone ID: V3LHS_314009), and nonsilencing negative control shRNA (catalog no. RHS4348) were purchased from Thermo Fisher Scientific (Lafayette, CO). Puromycin was purchased from Cellgro (Manassas, VA). Mouse anti-KRAS, rabbit antiphospho-ERK1/2 (Thr202/Tyr 204), phospho-AKT, and rabbit anti-p21 were purchased from Santa Cruz Biotech. Inc. (Santa Cruz, CA). Mouse anti-BCL2 was purchased from BD Biosciences Inc. (San Jose, CA). Mouse anti- β -ACTIN was purchased from Sigma-Aldrich (St. Louis, MO). Horseradish peroxidase-conjugated goat secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA), and Bradford Protein Assay came from Bio-Rad Laboratories (Hercules, CA). The Human Cancer Pathway-Focused PCR Array, miScript SYBR Green PCR Kit, and miScript Primer Assays for miR-134-5p, miR-373-3p, miR-205-5p, miR-155-5p, and RNU6-2 were purchased from Qiagen Inc. (Valencia, CA).

Cells and Cell Culture

Cd-transformed prostate epithelial (CTPE) cells were originally developed from continuous exposure of immortalized nontumorigenic human prostate epithelial cells, RWPE-1 to 10 μ M Cd for 8 weeks.⁴ The transformed CTPE cells showed loss of contact inhibition, increased secretion of matrix metalloproteinase-9 (MMP-9) and MMP-2 *in vitro*, and produced highly aggressive tumors upon inoculation in nude mice. CTPE cells were grown in K-SFM medium containing 50 μ g/mL BPE and 5 ng/mL EGF, supplemented with 1% antibiotic/antimycotic mixture. The cells were incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide. Culture of CTPE cells in Cd-free medium does not reverse their malignant phenotype.^{4,15}

Transduction of Lentiviral Particles into Cd-Transformed Cells

Studies to achieve a stable KD of KRAS in Cd-transformed cells were designed using transduction of KRAS shRNAir, which primarily expresses miRNA 30 transcript which targets KRAS protein and transcript to silence expression. Cd-transformed CTPE cells were seeded at a density of 150 000 cells in 6-well plates and allowed to attach overnight. Cells were transduced with lentiviral particles carrying the KRAS shRNAir, or nontargeting negative control shRNAir, at a multiplicity of infection (MOI) of 10. Cells were monitored after 24 h for green fluorescence protein (GFP) expression. The medium was removed, and

cells were grown in K-SFM medium for another 24 h. Puromycin (3 µg/mL) was added to media to select for stable cells, which were then harvested and maintained in selection media, and analyzed for gene KD 2 weeks after transduction. A stable KRAS KD phenotype was maintained throughout the experiment (up to 12 weeks) by inclusion of puromycin (3 µg/mL) in culture media. *In vitro* biomarkers of malignant phenotype were assessed every 3 weeks to determine how the loss of KRAS overexpression might impact the malignant phenotype of these Cd-transformed cells.

Western Blot Analysis

Whole cell lysates were obtained from either transformed CTPE cells, KRAS KD CTPE cells, or their nonsilencing negative controls for Western blotting as described in prior work with arsenic transformants.¹³ Antibodies used were to detect KRAS, phospho-ERK1/2, phospho-AKT, p21, BCL2, and β-ACTIN (as the loading control). Densitometric analysis was performed using Quantity One software (Bio-Rad).

MMP Activity

Increased secreted MMP activity generally correlates well with Cd-induced malignant transformation.^{4,16–18} After stable KD of KRAS, cells were plated in 6-well plates and grown to collect 48-h conditioned medium (without growth factors). The activity of MMPs in the conditioned medium was examined by zymography, as previously described.¹⁹

Anchorage-Independent Growth

Anchorage-independent growth was assessed by colony formation in soft agar as previously described.¹⁹

Cell Proliferation

We assessed cell proliferation in CTPE cells using a Cell Titer 96 Aqueous One Solution Cell proliferation Assay (MTS) kit (Promega, Madison, WI) with cells seeded in 96-well plates, according to manufacturer's instructions. We also assessed cell proliferation by seeding cells in 6-well plates, and allowing the cells to grow for 7 days, after which the cells were harvested and counted with trypan blue staining.

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNAs, including miRNAs, were isolated from either transformed CTPE cells, or KRAS KD CTPE cells, or its silenced control cells using miRNeasy kit (Qiagen Inc., Valencia, CA) according to manufacturer's instructions. The concentration of total RNA was evaluated using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Rochester, NY).

For the analysis of miRNA expression, cDNA was generated from RNA by the miScript II RT kit (Qiagen Inc., Valencia, CA) according to manufacturer's instructions. For miRNA profiling of transformed CTPE cells (before KRAS KD), the resulting cDNA was used as the template for real-time PCR with the miScript SYBR Green PCR Kit and the Human Cancer Pathway-Focused PCR Array (Qiagen Inc., Valencia, CA) following the manufacturer's instructions. The PCR array contains miRNA primers that profile the

expression of 84 miRNAs that are differentially expressed in tumor versus normal tissues (Qiagen Inc., Valencia, CA). Six small RNAs, SNORD 61, 68, 72, 95, 96A, and RNU6B/RNU6-2 served as internal controls, the average of which was used to normalize data. Data were analyzed using the $\Delta\Delta C_t$ method of relative quantification in which cycle times (C_t) of miRNAs were first normalized to that of the average of the internal controls and then to the passage-matched controls. Fold regulation is compared to miRNA expression in RWPE-1 cells. The expression of selected miRNAs was also analyzed in the CTPE cells following KRAS knockdown. In this way, cDNA was used as the template for real-time PCR with the miScript SYBR Green PCR Kit and miScript Primer Assays for miR-134-5p, miR-373-3p, miR-205-5p, miR-155-5p, and RNU6-2 (Qiagen Inc., Valencia, CA) following the manufacturer's instructions. Cycle times were normalized with RNU6-2 internal control and then expressed as a percentage of nonsilencing negative control.

For the analysis of mRNA expression, RNA was reverse transcribed with Moloney murine leukemia virus (MuLV) reverse transcriptase and oligo-d (T) primers. The primers for selected genes were designed with ABI Primer Express 3.0 Software (Applied Biosystems, Foster City, CA). Absolute SYBR Green ROX Mix (ThermoFisher Scientific, Rochester, NY) was used for RT-PCR analysis. Data were analyzed using the $\Delta\Delta C_t$ method of relative quantification in which cycle times were normalized with GAPDH from the same sample and then expressed as a percentage of nonsilencing negative control. Primer sequences are shown in Supplemental Table 1.

All real-time fluorescence detection was done on an iCycler (Bio-Rad, Hercules, CA).

Statistical Analysis

All data represent the mean $\pm$ SEM from three or more independent experiments. Statistical analyses were performed using an unpaired Student's *t*-test. A two-tailed $p < 0.05$ was considered significant in all cases.

RESULTS

KRAS Expression and Signaling in Cd-Transformed Prostate Cells

KRAS is an oncogene that belongs to the RAS superfamily of small GTPases and is often overexpressed in cancers, suggesting a key role in carcinogenesis. KRAS was overexpressed in arsenic-transformed prostate epithelial cells (CAsE-PE), and our recent work showed that KRAS silencing mitigated the malignant phenotype of these cells, strongly suggesting a key role for this gene in arsenic-induced malignant transformation.¹³ KRAS overexpression has also been reported in Cd-transformed human lung epithelial cells,¹⁷ thereby suggesting a likely role in Cd carcinogenesis, at least in other target cells. In the present work, we found KRAS activation was also associated with Cd-induced malignant transformation of prostate epithelial cells (CTPE cells). Indeed, as seen in Figure 1A, the CTPE cells greatly overexpressed KRAS protein compared to passage-matched nontransformed RWPE-1 controls (20-fold). This strongly suggests that activation of this oncogene likely plays a key role in Cd transformation of these cells.

The KRAS protein is located at a signaling node that is activated in response to multiple extracellular signals and interacts with an array of downstream effectors, to transduce signals leading to altered gene expression.⁹ For instance, the RAS/ERK signaling pathway plays a key role in cell survival, differentiation, proliferation, angiogenesis, motility, and metabolism, and if dysregulated, it promotes oncogenic transformation and progression.⁹ Indeed, the RAS/ERK pathway is activated in arsenic-transformed prostate epithelium, CAsE-PE cells.¹¹ Whether or not the RAS/ERK pathway activation also occurs in the prostate cells transformed by Cd was thus determined. Analysis of ERK showed increased expression of total ERK (216% control) and phospho-ERK (415% control) in CTPE cells (Figure 1B). Thus, Cd induced marked activation of this pathway in these cells.

Stable KD of KRAS in Cd-Transformed Prostate Cells

On the basis of initial findings, suggesting a likely role of KRAS in Cd transformation of these cells, we used a lentiviral shRNAmir technique to obtain specific and stable KD of KRAS, in order to determine if KRAS is critical in stimulating and maintaining malignant phenotype in the CTPE cells. The KRAS protein decreased by at least 99% in the KD CTPE cells compared to transformed (nontargeting or transformed control) control (Figure 2A). KRAS mRNA decreased by 45% (Figure 2B). This indicates the miRNA 30 shRNA was more effective at inhibiting protein translation than degrading mRNA, but in any event, it effectively silenced KRAS production. There was no difference in KRAS protein levels comparing nontargeted control with untransduced cells, indicating viral transduction was nontoxic. Thus, we were able to isolate clones in CTPE cells with stable loss of KRAS protein expression using the shRNAmir system. To stably maintain the phenotype, puromycin was continuously included in the media through the remainder of the experiment.

Stable KRAS KD Reduces the RAS/ERK Signaling Pathway

After establishing stable KRAS KD, we assessed various molecular and physical characteristics associated with the Cd-induced oncogenic phenotype in the CTPE cells. In this regard, we observed that KRAS KD caused changes downstream the MAPK signaling pathway. As shown in Figure 2C, KRAS KD decreased the level of phosphorylated ERK1/2 (p-ERK1/2) protein by 55% in the CTPE transformants, indicating a partial inhibition of the stimulated RAS/ERK signaling pathway. The PI3K/AKT pathway, which is also commonly activated by KRAS, was repressed by KRAS KD as seen by the marked decrease in phosphorylated AKT protein level (by 68%), a key effector molecule in the PI3K/AKT pathway (Figure 2C).

Stable KRAS KD Decreases Secreted MMP Activities

Elevated secreted matrix metalloproteinase (MMP) enzyme activity is typical of cancer cells. In the previous study of Cd-induced malignant transformation, CTPE cells showed increased activity of secreted MMP-2 (2.4 fold) over control and produced xenograft mice tumors.⁴ Thus, we determined the effect of KRAS KD on secreted MMP enzyme activity. As would be expected, KRAS KD consistently decreased elevated secreted MMP-2 enzyme activity in CTPE cells (by between ~30–70% of transformed control) as early as 3 weeks and persisted through 12 weeks (Figure 3), indicative of loss of the cancer phenotype. The reasons for the erratic nature of the decreases in MMP secretion in KRAS KD cells are unclear, but

secretion was consistently reduced at all times measured. The loss of 75% in the KRAS KD cells compared with CTPE (transformed control) cells was equal to approximately 120% of the MMP-2 activity in parental (control) RWPE-1 cells (data not shown), suggesting KRAS KD only partially mitigates the cancer phenotype of CTPE cells. This partial reversal of MMP secretion toward normal by KRAS KD was also observed in As-CSC cells.¹³

Stable KRAS KD Suppresses Colony Formation and Cell Proliferation

Anchorage-independent growth, as assessed by colony formation, is also common for cancer cells and indicates aggressive phenotype. KRAS KD led to a significant decrease (by 52%) in colony formation (Figure 4A) as early as 6 weeks post transduction and persisted through 12 weeks, indicative of perturbed cancer phenotype. We also assessed the effect of KRAS KD on cell proliferation in the CTPE cells. At 12 weeks post transduction, KRAS KD reduced proliferation of the CTPE cells over 1 week as assessed by both the vital stain trypan blue (not shown) and the MTS assay (Figure 4B).

Decreased cell proliferation in KRAS KD cells was accompanied by significant increases in p21 protein (342% of transformed control) and p16 (16%), and p27 (27%) transcripts (Figure 4C,D, respectively), which are genes that inhibit cell cycle. The restoration of p27 transcript (which was initially repressed at transformation (Figure 6A)) by KRAS KD indicates a diminution of the malignant phenotype.

Effects of KRAS KD on Other Cancer-Related Genes

KRAS KD decreased BCL2 protein levels (by 61%) (Figure 5A) and *BCL-w* transcript (by 10%) (Figure 5B), both of which are antiapoptotic genes that favor cell survival. KRAS KD also restored the expression of *PTEN* (Figure 5B), which was originally repressed by Cd transformation (Figure 5C). The decreased cell survival and restoration of tumor suppressor gene, PTEN of cancer cells, are consistent with loss of malignant phenotype.

miRNA Profiling and Target Gene Expression

We used qRT-PCR to determine the expression levels of 84 human mature miRNAs (that are differentially expressed in tumor versus normal tissues) in the Cd-transformed cells in order to determine whether miRNAs play a role in Cd transformation. Of the 84 miRNAs profiled, 15 miRNAs were differentially expressed by 1.5 fold over control in CTPE cells (Table 1). There was significantly more down-regulated (12 miRNAs) than up-regulated (3 miRNAs) miRNAs in the transformants. These data suggest aberrant miRNA expression might be significant in the malignant transformation process of prostate epithelium by Cd.

It is thought miRNAs regulate expression of target genes by mRNA degradation or translational repression. We used the bioinformatics tools, TargetScan 6.2 (<http://www.targetscan.org>) and microRNA.org (<http://www.microrna.org>), which are available online, to identify cancer-related gene targets of selected dysregulated miRNAs in the Cd transformants. Predicted or previously confirmed targets were directly analyzed by gene expression (mRNA and/or protein production). As expected, expression of dysregulated miRNAs (Table 1) inversely correlated with target gene expression at the transcript and/or protein level in the Cd transformants (Figure 1A, 6A,B). Altered gene targets include: (i)

increased KRAS mRNA by 2000% and RAB22A mRNA by 48% (oncogenes); (ii) increased E2F1 mRNA by 52% (cell signaling); (iii) decreased CADM1 mRNA by 65% and CTNNA1 mRNA by 25% (cell adhesion related genes); and (iv) increased BCL2L1 mRNA by 25%, decreased p27^{Kip1} protein by 49% and FOXO4 mRNA by 61% (cell survival and apoptosis-related genes). The miRNA changes in CTPE cells would generally favor tumor formation, suggesting a key but complex role in the malignant transformation of prostate epithelial cells by Cd.

Effects of Stable KRAS KD on miRNA Expression

On the basis of initial findings that miRNA dysregulation was associated with, and likely plays a role in, Cd transformation of the prostate cells, we determined the effect of KRAS KD on selected dysregulated miRNAs in these transformants. The goal was to determine if the expression of the dysregulated miRNAs would be reversed as the cells lose their cancer phenotype following KRAS KD. We observed that KRAS KD did not reverse the expression of selected miRNAs that were dysregulated at transformation. Instead, KRAS KD increased the expression of miR-134 by 75%, and decreased miR-155 (by 45%) and miR-205 (by 17%) (Supplemental Figure 1), which were all changed in the same direction at transformation.

DISCUSSION

Although the correlation between cadmium (Cd) exposure and the risk of prostate cancer is inconsistent, epidemiological studies have seen positive associations between Cd exposure and prostate cancer.² These associations make the prostatic epithelium a suspected target of cadmium although the mechanisms involved in transformation are not clearly defined. In the present work, the KRAS oncogene was identified as critical to the malignant transformation of CTPE cells, prostate epithelial cells that acquired a malignant phenotype *in vitro* through chronic Cd exposure.⁴ These cells were observed to greatly overexpress KRAS, which activated the downstream RAS/ERK signaling pathway, factors known to favor malignant tumor formation.⁹ The key role of KRAS in causing and maintaining this Cd-induced malignant phenotype was demonstrated by using a shRNA technology to stably KD and silence the expression of *KRAS*. KRAS KD clearly diminished multiple physical and molecular aspects of the CTPE cell cancer phenotype. This includes reduced secreted MMP enzyme activity, reduced colony formation, reduced cell proliferation, and realignment toward the normal of expression of genes linked to proliferation, cell survival, and tumor suppression. All these factors support a reduction of malignant phenotype, they and are consistent with multiple reports showing that KD of wild-type or mutant *KRAS* can reduce cancer phenotype *in vitro*¹³ and decrease *in vivo* tumorigenicity potential of pancreatic cancer,^{20–22} colorectal cancer,²³ and nonsmall cell lung cancer²⁴ cells. However, KRAS is not the only genetic factor associated with Cd-induced malignant transformation, as it is clear that KRAS KD did not totally reverse Cd-induced malignant phenotype.

KRAS is not only commonly activated in advanced prostate cancer cells, as seen in the present work but may be a critical factor that originally drives prostate cancer initiation.¹⁴ In prior work using RWPE-1 cells transformed by inorganic arsenic (CAsE-PE), a potential

human prostatic carcinogen,² activation of KRAS was pronounced and showed a key role in causing and maintaining the malignant phenotype.¹³ In the present study with RWPE-1 cells malignantly transformed by Cd (CTPE cells), we observed a similar overexpression of KRAS, suggesting a role of KRAS in Cd-induced prostate carcinogenesis, consistent with arsenic. It is important to note that KRAS activation is not obligatory to the malignant transformation of RWPE-1 cells, as suggested by the findings that WPE1-NB26 cells, which are RWPE-1 cells transformed by *N*-methyl-*N*-nitrosourea (MNU),²⁵ a direct genotoxin, show no evidence of enhanced KRAS signaling (unpublished observation). Thus, KRAS activation in the transformed RWPE-1 cells is dependent on the carcinogen. Our current findings are consistent with other studies which report KRAS activation in Cd- and arsenic-transformed human lung cells,^{17,26} suggesting KRAS activation appears to be an important event for both of these inorganic carcinogens. Interestingly, the lung is a human target tissue for both Cd and arsenic.²

KRAS activation often occurs in cancers through activating mutations in the KRAS gene.¹⁰ However, it is also clear that increased expression of the wild-type (i.e., nonmutated) KRAS is a significant factor in generating malignancies, including prostatic malignancies,^{14,17,27} and arsenic-transformed prostate cells.¹¹ In the current study, it is unknown whether the overexpressed KRAS in the Cd-transformed prostate cells (CTPE) is wild type or mutant. However, the system used in the present study would have resulted in KD of nonmutant KRAS in the CTPE cells, and this resulted in markedly mitigated cancer phenotype. These results are similar to those obtained from loss of tumor qualities after KD of the mutant KRAS in pancreatic, lung, and colorectal tumors.^{20–24} Furthermore, with respect to a complex set of genetic alterations, it is considered that enhanced KRAS signal transduction is one of the most common events in prostatic oncogenesis²⁸ regardless of the original stimulus for KRAS activation. The reduced cancer phenotype observed from the KD of nonmutant KRAS in the current study thus strongly suggests that the reversal of the overexpressed KRAS induced by Cd transformation played a fundamental role in reversing oncogenic phenotype. These findings are similar to those previously observed in the arsenic-transformed prostate cells¹¹ and prostate stem cells¹³ which overexpressed the wild-type KRAS and showed reduced cancer phenotype following KD of nonmutant KRAS,^{11,13} suggesting that arsenic and Cd share, at least in part, some commonality in mechanisms of prostatic epithelial cell transformation.

MMPs are secreted enzymes that selectively degrade the extracellular matrix and basement membranes. MMPs have been implicated in tumor progression,²⁹ tumor angiogenesis,³⁰ cancer cell metastasis and migration.^{31,32} MMPs also regulate signaling pathways by activating specific receptors or growth factors resulting in inflammation, cell growth, or angiogenesis, and they may even work in a nonproteolytic manner.³³ There are clear correlations between aggressive tumorigenesis or cellular malignant transformation and hypersecretion of various MMPs, including MMP-2.^{4–6,16–18,34} For example, hypersecretion of MMPs highly correlated with the malignant transformation and invasiveness of Cd-transformed prostate cells.⁴ Hence it would be expected that a loss in cancer phenotype would be associated with reduction in secreted MMP activity. As expected, KRAS KD markedly decreased secreted MMP-2 activity in the CTPE cells, pointing toward a marked decrease in cancer phenotype.

Cancer cells are typically characterized by aberrant or rapid proliferation.³⁵ Thus, a loss in cancer phenotype should reduce proliferation rate. We showed that KRAS KD of Cd-transformed CTPE cells decreased cell proliferation, indicating a diminished cancer phenotype. Colony formation in soft agar, an indication of anchorage-independent growth, which is a characteristic common for cancer cells, was also markedly decreased following KRAS KD in the Cd-transformed CTPE cells, indicating reduced cancer phenotype. These findings are consistent with reports of decreased anchorage-independent growth in arsenic-transformed prostate epithelial and stem cells following nonmutant KRAS KD,¹³ and in the human pancreatic cell line CAPAN-1 following KD of mutant *K-RAS*^{V12}.³⁶

miRNAs are small noncoding RNAs that negatively regulate gene expression. They bind to the 3' untranslated region (3'-UTR) of their target mRNA resulting in translational repression or degradation of mRNA.³⁷ miRNAs regulate many cellular processes, and alterations in their expression can facilitate development of cancer, including prostate cancer.^{12,38,39} It is a distinct possibility that the miRNA expression profile of prostate cancers induced by different carcinogens could be unique to the individual carcinogen. Thus, determining the role of miRNAs in Cd-induced prostatic malignant phenotype may help provide insights into the underlying molecular mechanisms of Cd transformation. Our results revealed a distinct miRNA expression signature in the Cd-transformed prostate cells, and in many cases, the miRNA dysregulation correlated with expression of target genes, such as miR-155, miR-205, and miR-134. These data suggest miRNA dysregulation is likely important in Cd-induced malignant transformation and are consistent with reports of altered miRNA expression playing a pathogenic role in human prostate carcinogenesis.^{12,40} There was a similarity in the miRNA signature between Cd-transformants in the current study and arsenic-transformants in our previous study,¹³ in that 80% of the dysregulated miRNAs in the Cd-transformants were also dysregulated in the same direction in the arsenic-transformants. For example, miR-96 and miR-9 were upregulated in Cd- and arsenic transformants, whereas miR-205, miR-155, miR-373, miR-125a-5p, miR-146b-5p, miR-138, miR-222, miR-181d, let-7b, and let-7c were downregulated in both transformants. These data strongly suggest both carcinogens share some common mechanistic pathways like oncogenesis, cell survival, and altered apoptosis and cell adhesion. However, KRAS KD in the Cd-transformants did not reverse the expression of the dysregulated miRNAs that might control KRAS expression. One would expect a reversal in the expression of these KRAS-relevant miRNAs as the KRAS KD Cd-transformants lose their malignant phenotype. This reversal in KRAS-relevant miRNA expression has been demonstrated previously during KRAS KD and mitigation of cancer phenotype of isogenic prostate epithelial and stem cells malignantly transformed by arsenic.¹³ It is unclear why the KRAS-relevant miRNA expression was not similarly reversed as the KRAS KD Cd-transformants lost their malignant phenotype. This strongly suggests that there is a complex and perhaps indirect relationship between Cd transformation, specific miRNAs, and KRAS overexpression, at least in the prostate. These data point toward a major difference in molecular mechanisms between Cd- and arsenic-induced carcinogenesis in the prostate, although both can be manipulated by KRAS expression.

CONCLUSIONS

Our data provide evidence that malignant transformation of prostate epithelial cells by Cd likely involves, at least in part, overexpression of the KRAS oncogene, which is thought to be a key factor in prostate cancer formation and progression. The Cd-induced malignant phenotype was largely mitigated by KD of KRAS, as shown by the marked reduction of multiple physical and molecular characteristics of prostate cancer cell phenotype. These data strongly suggest that KRAS is a key gene for Cd-induced malignant phenotype in the prostate. However, KRAS is not the only genetic factor sustaining malignancy in these Cd-transformed cells, as it is clear that KRAS KD did not totally reverse Cd-induced malignant phenotype. Further research is needed to define other critical aspects involved in Cd-induced malignant transformation of these prostate cells. This work is now being extended to other cell models of Cd carcinogenesis, such as the lung where KRAS likely plays a major role.¹⁷ This additional work will be crucial to help confirm the importance and determine the precise role of KRAS in Cd-induced malignant transformation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Cd	cadmium
RWPE-1	normal human prostate epithelial
CTPE	cadmium-transformed prostate epithelial
CAsE-PE	arsenic-transformed prostate epithelial
As-CSCs	arsenic-transformed prostate cancer stem cells
SCs	stem cells
KD	knockdown
MMP	matrix metalloproteinase
K-SFM	kerati-nocyte serum-free medium
BPE	bovine pituitary extract
EGF	epithelial growth factor

shRNAmir	short hairpin miRNA
GFP	green florescent protein
MTS	cell titer 96 aqueous one solution cell proliferation assay
RT-PCR	reverse transcriptase polymerase chain reaction
MNU	<i>N</i> -methyl- <i>N</i> -nitrosourea
CAPAN-1	a human pancreatic cell line

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Figure 1. Analysis of KRAS expression and signaling in CTPE cells. (A) KRAS protein levels; (B) Protein levels of ERK and p-ERK (activated form) in the RAS/ERK signaling pathway. Data for the analysis of the RAS/ERK pathway represent mean $\pm$ SEM ($n = 3$). * $p < 0.05$ compared with controls.

Figure 2.

Transduction of CTPC cells with KRAS shRNA or nonsilencing negative control shRNA (sham). (A) KRAS protein levels in untransduced cells, transformed control (sham) cells, and KRAS KD; (B) KRAS transcript levels compared to sham shRNA or untransduced cells; (C) Analysis of the RAS/ERK and PI3K/AKT pathway in transformed control (sham shRNA) or *KRAS* knockdown cells. Data for the analysis of the RAS/ERK and PI3K/AKT pathway represent mean $\pm$ SEM ($n = 3$). * $p < 0.05$ compared with controls.

Figure 3. Analysis of secreted MMP-2 enzyme activity in CTPE cells after KRAS knockdown. Enzyme activity of sham control was set at 100%. Inset: representative zymogram of secreted MMP-2 at 12 weeks after transduction. Data represent mean $\pm$ SEM ($n = 3$). * $p < 0.05$ compared with controls.

Figure 4. Effects of KRAS knockdown on *in vitro* cell growth of CTPE cells. (A) Colony formation in soft agar. (B) CTPE cell proliferation as assessed by MTS assay and cell count at 1 week. (C) Protein level of p21. (D) Transcript levels of *p16* and *p27*. Data represent mean $\pm$ SEM ($n = 6$, except for cell count where $n = 3$). * $p < 0.05$.

Figure 5. Effects of KRAS knockdown on other cancer-related genes. (A) Protein level of BCL2. (B) Transcript levels of PTEN and BCL-w. (C) Protein level of PTEN before KRAS knockdown, i.e. at transformation. Data represent mean $\pm$ SEM ($n = 3$). * $p < 0.05$.

Figure 6.

Analysis of selected miRNA gene targets in CTPE cells at transformation, i.e. before KRAS knockdown. (A) Protein level of p21^{Kip1}, a miR-96 target. (B) Transcript levels. Data represent mean $\pm$ SEM ($n = 3$). * $p < 0.05$.

Table 1

Differentially expressed microRNAs in CTPE cells analyzed by microRNA cancer array

microRNA	fold regulation- n ^a (2 ^{- Ct})		predicted targets assayed ^b
	up	down	
miR-125a-5p		2.3	
miR-96	2.2		p27 ^{kip1} , FOXO4
miR-222		3.3	
let-7b		1.8	BCL2L1, RAS
miR-205		4.4	E2F1
miR-20a		2.6	
miR-146b-5p		5.2	
miR-134	2.7		CADM1
miR-138		2.4	RAB22A
miR-373		11.0	RAB22A, E2F1
let-7c		1.5	BCL2L1, RAS
miR-181d		1.8	KRAS
miR-155		5.2	KRAS
miR-9	5.9		CTNNA1
miR-149		1.7	

^aFold regulation is compared to microRNA expression in RWPE-1 cells and are significantly different ($p < 0.05$).

^bPredicted targets by TargetScan 6.2 and miRNA.org assayed; miRNA expression is inversely correlated with expression of target genes.