Published in final edited form as:

Chem Res Toxicol. 2013 January 18; 26(1): 96–105. doi:10.1021/tx3004054.

Chronic Exposure of Renal Stem Cells to Inorganic Arsenic Induces a Cancer Phenotype

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Abstract

Inorganic arsenic in the drinking water is a multisite human carcinogen that potentially targets the kidney. Recent evidence also indicates that developmental arsenic exposure impacts renal carcinogenesis in humans and mice. Emerging theory indicates that cancer may be a disease of stem cells (SCs) and that there are abundant active SCs during early life. Therefore, we hypothesized that inorganic arsenic targets SCs, or partially differentiated progenitor cells (PCs), for oncogenic transformation. Thus, a rat kidney SC/PC cell line, RIMM-18, was chronically exposed to low-level arsenite (500 nM) for up to 28 weeks. Multiple markers of acquired cancer phenotype were assessed biweekly during arsenic exposure, including secreted matrix metalloproteinase (MMP) activity, proliferation rate, colony formation in soft agar, and cellular invasiveness. Arsenic exposure by 10 weeks and after also induced marked and sustained increases in colony formation, indicative of the loss of contact inhibition, and increased invasiveness, both cancer cell characteristics. Compared to the passage-matched control, chronic arsenic exposure caused exposure-duration dependent increases in secreted MMP-2 and MMP-9 activity, Cox-2 expression, and more rapid proliferation (all >2-fold), characteristics typical of cancer cells. Dysregulation of SC maintenance genes and signaling pathways are common during oncogenesis. During arsenite exposure, expression of several genes associated with normal kidney development and SC regulation and differentiation (i.e., *Wt-1*, *Wnt-4*, *Bmp-7*, etc.) were aberrantly altered. Arsenic-exposed renal SCs produced more nonadherent spheroid bodies that grew much more aggressively in Matrigel, typical of cancer SCs (CSCs). The transformed cells also showed gene overexpression typical of renal SCs/CSCs (*CD24*, *Osr1*, *Ncam*) and arsenic adaptation such as overexpression of *Mt-1*, *Mt2*, *Sod-1*, and *Abcc2*. These data suggest that inorganic arsenic induced an acquired cancer phenotype *in vitro* in these rat kidney SCs potentially forming CSCs and, consistent with data *in vivo*, indicate that these multipotent SCs may be targets of arsenic during renal carcinogenesis.

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Supporting Information List of primers and their sequences. This material is available free of charge via the Internet at <http://pubs.acs.org>.

The authors declare no competing financial interest.

INTRODUCTION

Millions of people are exposed to inorganic arsenic in their drinking water at potentially unhealthy levels, making this metalloid a worldwide public health problem.¹ Exposure to inorganic arsenic is carcinogenic in humans with multiple target sites, potentially including the kidney.^{1–3} Early life exposures to inorganic arsenic appear to be particularly potent in cancer causation. For instance, in humans, Yuan et al.⁴ found elevated exposure to inorganic arsenic in the drinking water during early life resulted in markedly higher mortality from renal cancer in young adults. In mice, inorganic arsenic is an effective transplacental carcinogen at various sites, including the lung and liver.^{5,6} *In utero* inorganic arsenic exposure in mice also induces renal hyperplasia in the offspring during adulthood, long after all intentional exposure to the metalloid would have ended.⁶ The methylated metabolite of inorganic arsenic, dimethylarsinic acid (DMA), can cause uroepithelial tumors in adult rats.^{7,8} A recent model shows that mice develop renal cell carcinoma (RCC) as adults if exposed to inorganic arsenic *in utero* followed by DMA during adulthood,⁹ which is important as it duplicates a potential human target tissue¹ using only arsenicals. These studies, where early life arsenic exposure impacts renal cancer development or stimulates kidney proliferative lesions including tumors, much later in life, suggest a long-lived common stem/progenitor cell (SC/PC) phenotype may be targeted for carcinogenic transformation.^{4,6,9}

Indeed, increasing evidence suggests that cancer may often be a SC-based disease. The driving force behind the process of carcinogenesis is believed to be cancer SCs (CSCs), which are thought to arise from normal SCs, or closely differentiated progenitor cells (PCs).^{10,11} In this regard, arsenic has been shown to disrupt SC population dynamics and target these cells during arsenic-induced malignant transformation leading to an overabundance of CSCs both *in vitro* and *in vivo*.^{12–15} Moreover, in humans and rodents, the propensity that arsenic has for carcinogenic activity during early life^{4–6,16} coincides with a time of high activity for SCs.^{6,17} Indeed, SCs in the developing animal are suspected to be a critical point of attack during chemical carcinogenesis^{5,6,17} making these cells, which are also abundant during *in utero* life, likely targets for arsenic-induced developmental carcinogenesis.⁶ In this regard, the latency between early life arsenic exposure and eventual formation of kidney cancer in adulthood in humans⁴ or mice⁹ may be due to a targeting of a

conditionally immortal SC/PC population by inorganic arsenic. On the basis of these accumulating data, we hypothesized that arsenic may target and alter renal SCs/PCs during early development, essentially ‘priming’ them for oncogenesis later in life.

The development of models of arsenic-induced kidney cancer is crucial for defining the effect of the metalloid in this tissue, and models are needed at all levels of biological complexity. Thus, in the current study, the effects of low-level, chronic arsenic exposure were examined in the RIMM-18 cell line, developed as a rat renal SC/PC line.¹⁸ RIMM-18 cells were isolated from primary metanephric mesenchyme and transfected with an E1A-ER vector for immortalization.¹⁸ The metanephric mesenchyme contains kidney stem/progenitor cells.^{19,20} RIMM-18 cells were exposed to inorganic arsenic to potentially induce acquisition of characteristics that would be consistent with cancer cells, qualifying them as CSCs, to help fortify other studies indicating that SCs/PCs are targets for arsenic-induced transformation or tumor formation in various target sites like the skin and prostate.^{6,12,13,15}

MATERIALS AND METHODS

Chemicals and Reagents

Sodium arsenite, β -estradiol (E2), and *p*-iodonitrotetrazolium (INT) were obtained from Sigma (St. Louis, MO) and recombinant bovine fibroblast growth factor (FGF) from R&D Systems (Minneapolis, MN).

Cells, Culture Conditions, and Arsenite Exposure

Rat Inducible Metanephric Mesenchyme-18 (RIMM-18) is an immortalized SC/PC line established by transfection of primary rat metanephric mesenchyme cells with an E1A-ER vector.¹⁸ Arsenic exposure did not have any effect on ER- α levels in this study. Cells were grown in DMEM/F12 medium (Gibco/Invitrogen, Rockville, MD) containing 5% fetal bovine serum (FBS), 10 ng/mL FGF, and 100 nM E2. Preconfluent cells were subcultured once per week and maintained in a humidified atmosphere at 37 °C and 5% CO₂. Treated cells were continuously exposed to a nontoxic, low-level of arsenic (as sodium arsenite; 500 nM), and untreated time-matched control cells were grown concurrently for the duration of the experiments. Culture medium was refreshed every 3–4 days. Arsenic-containing medium was prepared fresh each time the medium was changed. Three separate flasks for control and arsenic-treated cells were maintained throughout. Rat kidney cells have been shown to have arsenic biotransformation ability,²¹ so it is possible that methylated metabolites could be acting on the RIMM-18 cells. However, the arsenic biotransformation capacity of RIMM-18 cells is unknown and whether the transformative agent was inorganic arsenic, a methylated metabolite, or some combination remains to be determined.

Cell Viability and Proliferation Assays

For both the viability assay and proliferation assay, cells (1.0×10^4 /well; $n = 3$) were plated in 6-well plates. For the viability assays, cells were allowed to attach overnight before exposing to the indicated concentrations of arsenic for 72 h to determine the lethal concentration in 50% (LC₅₀) of the cells. For the proliferation assay, every two weeks, control and arsenic-exposed cells were plated and allowed to grow for 72 h under normal culture conditions, and proliferation was measured. For both assays, at the point of assessment wells were washed 2 $\times$ with PBS, lifted with brief exposure to trypsin/EDTA/PBS (1:1 v/v), and cell viability/proliferation was measured using a standard trypan blue dye exclusion method and hemocytometer. Quantitation was based on untreated control cells being set at 100%.

Zymography for MMP Activity

Elevation of secreted matrix metalloproteinases (MMPs) is a common characteristic of cancerous cells. Secreted MMP-2 and MMP-9 activity was measured as described.¹³

Agar Assay

Colony formation in soft agar was conducted as described¹³ and used to measure the ability of cells to grow in an anchorage-independent manner, a common characteristic of cancer cells. Triplicate dishes were prepared for control and treated cells at each time point.

Invasion Assay

Cellular invasiveness, a characteristic typical of cancer cells, was measured using a modified Boyden chamber method as described.¹³

Sphere Formation and Sphere Growth in Matrigel

Free-floating spheres of cells were formed by plating a single-cell suspension into ultralow adherence flasks (2×10^4 cells/T-75 flask; Corning Inc., Corning, NY). Cells were fed every 48 h. To avoid disruption of sphere formation during feeding, the medium was gently added to each flask, and no medium was removed. After 10 days, spheres were counted as previously described,¹³ and single spheres were isolated and plated into 48-well plates in a 1:1 (v/v) mixture of growth medium and Matrigel (200 μ L; BD Biosciences). Images of spheres were taken after one week in the Matrigel mixture. Spheres in Matrigel formed from both arsenic-treated and control nonadherent spheroid bodies could be serially passaged multiple times, indicating the ability for self-renewal typical of SCs or CSCs.

Real Time RT-PCR

Gene expression levels were measured by real-time reverse transcription–polymerase chain reaction (RT-PCR) analysis as described.¹⁴ Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA), purified with RNeasy mini kit columns (Qiagen, Valencia, CA), and reverse transcribed to cDNA with the use of MuLV reverse transcriptase (Applied Biosystems, Foster City, CA) and oligo-dT primers. Primers were designed with the use of Primer Express 3.0 software (Applied Biosystems) and Primer-BLAST software (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>). Primer specificity was examined by RT-PCR from rat embryonic kidney cDNA and a single peak in the dissociation curves. For primer efficiency, only those yielding a single band in gels following amplification were included in these studies. The ABsolute SYBR Green ROX Mix (ABgene, Rockford, IL) was used for amplifications. Cycle time (Ct) values for the selected genes were normalized to values for β -actin and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) in the same sample. Real time RT-PCR data were analyzed using the $\Delta\Delta C_t$ method. Three independent flasks ($n = 3$) for each treatment were collected, and Ct values for control cells were set at 100%. Primer sequences are listed in Supporting Information, Table S1.

Immunohistochemical Detection of CD133 in Renal Cell Carcinoma

Mice treated with inorganic arsenic *in utero* followed by DMA in adulthood developed RCC in excess.⁹ Paraffin-embedded sections (5 μ m) of representative RCC from these arsenical-treated mice were used for immunohistochemical analysis of CSCs in the tumors. RCCs from mice born to mothers that had been given 0.5 mmol/kg of *N*-nitrosoethylurea (ENU), s.c., on gestation day 16 were used for comparison. Localization and intensity of CD133, a kidney CSC marker, was studied in RCC using a polyclonal antibody to CD133 (1:400; Abcam, Cambridge, MA). Diaminobenzidine from Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) was used for final detection. To define specificity, the primary

antibodies were omitted from each staining series as a control. Three separate samples from different mice of arsenic-induced and ENU-induced RCC were assessed. All tissues were evaluated without prior knowledge of the treatment group. Brown areas indicate positive staining for CD133.

Statistical Analysis

All data except tumor incidence represent mean $\pm$ standard deviation (SD). An unpaired Student's *t* test was used to compare individual time-points of arsenic-treated cells to untreated time-matched controls. For the LC₅₀ curve, a Dunnett's *t* test was used after ANOVA to adjust for multiple comparisons. In all cases, a two-sided *p* < 0.05 is considered significant and is indicated by an asterisk (*).

RESULTS

Arsenic Cytotoxicity in Kidney Stem Cells

RIMM-18 cells were treated with a range of concentrations of sodium arsenite to determine the LC₅₀ of arsenite in these cells. The LC₅₀ concentration for RIMM-18 cells was calculated to be 13.6 μ M (Figure 1). These results were used to determine the arsenic concentration used for the chronic exposure experiments. On the basis of a complete absence of effects on viability or growth, 500 nM arsenite was the concentration selected for chronic exposure. This concentration is below levels previously shown to be environmentally relevant.²²

Arsenite-Induced Transformation of Kidney Stem Cells

MMPs are enzymes that help degrade the extracellular matrix and facilitate invasion and metastasis of malignant cells.²³ Increased secreted MMP-9 activity is a very consistent marker of arsenic-induced cell transformation.^{13,24–26} Compared with time-matched untreated controls, arsenite-treated RIMM-18 cells showed marked increases of 2–4-fold of the control in MMP-9 and MMP-2 by 28 weeks of exposure (Figure 2A). Secreted MMP activity in this range is consistent with malignant transformation by inorganic arsenic in various other cell lines,^{24–26} including SC lines.¹³

Anchorage-independent colony formation in soft agar is commonly used to measure the *in vitro* carcinogenic potential and often correlates with *in vivo* tumorigenicity.²⁷ As seen in Figure 2B, arsenite caused a dramatic increase in colony formation in these cells. A 9-fold increase occurred with arsenite compared with untreated controls by 10 weeks of exposure that was a maximal 12-fold increase by 28 weeks of exposure.

Invasive ability is a key characteristic of cancer cells and plays an important role in cancer progression, including local extension and metastasis. Arsenite treatment induced a marked time-dependent increase in invasive ability of RIMM-18 cells. Compared with untreated controls, arsenite-treated cells showed an approximate 2-fold increase in invasion by 28 weeks of exposure (Figure 2C).

Cyclooxygenase-2 (Cox-2) levels are increased in a wide variety of premalignant and malignant tissues, including RCC, and there is a considerable amount of evidence suggesting a relationship between increased Cox-2 activity and carcinogenesis.^{28,29} Chronic arsenic exposure increased Cox-2 expression in RIMM-18 cells nearly 7-fold compared to the control by 28 weeks of exposure (Figure 2D). This dramatic increase in Cox-2 fortifies the contention that an acquired cancer phenotype has been precipitated by chronic low-level arsenic exposure.

Compared with the control, arsenite-exposed cells showed a significant increase in the ability to form nonadherent spheres and in average sphere size (Figure 2E). When similar-sized spheres were plated in Matrigel, the arsenite-treated spheres rapidly formed large, branching, distorted ductal-like structures, while the spheres from control cells showed only modest signs of ductal formation in the same time period (Figure 2F). This suggests a much more “aggressive” phenotype in the spheres formed from arsenite-exposed SCs.

Arsenic Alters the Expression of Genes Linked to Normal Kidney Development and Kidney SCs

Many factors and signaling pathways act as critical regulators of kidney development and kidney SCs. During chronic arsenic exposure, the transcript levels of *Wnt4* showed an early decrease to ~45% of control but then showed a reactivation to approximately 40% over untreated controls by 28 weeks of exposure (Figure 3A). *β-Catenin*, a key component of the Wnt signaling pathway, showed a persistent decrease in expression during chronic arsenic exposure, down to ~50% of the control by 28 weeks (Figure 3B). Similarly, chronic arsenic exposure caused the bone morphogenic protein-7 (*Bmp-7*) transcript to decrease to ~50% of control levels at both 14 weeks and 28 weeks (Figure 3C). Similar to the U-shaped trend seen with *Wnt4* transcript levels, Wilms’ tumor protein-1 (*Wt-1*) transcript levels decreased to 50% of the control at 14 weeks of arsenic exposure but by 28 weeks *Wt-1* expression had increased to about 160% compared to that of the control (Figure 3D).

Arsenic exposure also markedly increased the expression of several other widely accepted kidney SC markers. *Cd24*, odd-skipped related 1 (*Osr1*), and *Cd133* all showed marked increases to 2.3-fold (Figure 4A), 3.5-fold (Figure 4B), and 3.2-fold (Figure 4D), respectively, of control cells by 28 weeks of arsenic exposure. The neural cell adhesion molecule (*Ncam*) increased almost 2-fold by 14 weeks (not shown) and further increased to 2.3-fold of the control by 28 weeks of arsenic exposure (Figure 4C).

In a mouse RCC induced by early life inorganic arsenic exposure followed by additional arsenical later in life,⁹ immunohistochemical analysis showed a widespread and focally intense presence of CD133 protein, another renal SC/CSC marker (Figure 4E). In the arsenical-induced kidney tumor (top), the renal SC/CSC marker was clearly overexpressed compared with that in an ENU-induced RCC (bottom) and showed intense staining in the prospective CSCs.

Kidney Stem/Progenitor Cells Adapt to Arsenic during Chronic Exposure

Several factors involved in arsenic adaptation increased in the RIMM-18 cells during chronic exposure. Metallothionein-1 (*Mt-1*), superoxide dismutase-1 (*Sod-1*), and ATP binding cassette transporter C2 (*Abcc2*) all showed only modest increases in transcript levels during early arsenic exposure (not shown) but significant increases by 28 weeks of exposure compared with that of the control (Figure 5). *Mt-2* showed marked early increases (~9-fold at 14 weeks; not shown) with a maximal increase of 11.5-fold by 28 weeks of exposure (Figure 5). Moderate increases were also seen in *Abcc1* (1.34-fold) and glutathione-S-transferase-pi (*Gst-pi*; 1.25-fold) by 28 weeks of exposure (not shown).

DISCUSSION

Kidney cancer is a common and often fatal malignancy.³⁰ Arsenic has long been recognized as a multisite human carcinogen, and it is now appreciated that it potentially targets the kidney.¹ However, animal models of arsenic-induced kidney cancer have been difficult to develop. Studies using various strains show adult rats exposed to inorganic arsenic alone during adulthood fail to develop kidney cancer, although inorganic arsenic or DMA can

promote organic carcinogen-initiated renal cancer.^{31,32} It was not until the development of a transplacental model of inorganic arsenic carcinogenesis^{5,6} that it was possible to create animal models of arsenic-induced renal cancer.⁹ In this latter study, mice that are exposed *in utero* to inorganic arsenic via the maternal drinking water, followed by DMA in the water during adulthood develop RCC.⁹ This is consistent with human data that associated early life arsenic exposure and high renal cancer mortality in young adults.⁴ The mouse early life inorganic arsenic exposure studies, coupled with the emerging evidence that arsenic targets stem cells (SCs) for transformation in multiple model systems,^{6,12–15} have begun to reveal possible reasons why rodent models of inorganic arsenic acting alone as a carcinogen in adulthood may have been difficult to develop. This could be, at least for some tissues, because arsenic targets SCs for transformation and exposure during the fetal/developmental life stage, which is a time when SCs are both abundant and highly active due to factors like organogenesis, global proliferative growth, and intensive cellular differentiation.^{6,17} The kidney may be one such target since mice developed tumors and renal hyperplasia with inorganic arsenic only if there was a component of exposure during early life.^{5,6,9} To fortify this hypothesis, the current study examined the effects of inorganic arsenic exposure directly on a rat kidney SC/PC line, RIMM-18. This line was derived from the metanephric mesenchyme,¹⁸ which is composed of renal SCs/PCs,^{19,20} making it ideal to test as a target for arsenic. Indeed, our data indicate that these renal SCs acquired multiple cancer cell characteristics during chronic inorganic arsenic exposure *in vitro*. This fortifies the concept that the SC phenotype is a key target cell in arsenic carcinogenesis in yet another cell type. Other work has shown that inorganic arsenic impacts or targets during cellular transformation human skin SCs^{15,25} and prostate SCs.^{13,14} Similarly, developmental inorganic arsenic in mice causes an apparent overabundance of CSCs in resultant carcinoma in the liver and lung,³³ and the kidney (present work). Prenatal inorganic arsenic exposure also predisposes Tg-AC mice to chemically induced squamous cell carcinoma (SCC) in adulthood that are highly enriched in CSCs compared to SCC developed without early life arsenic exposure.¹² Thus, a consistent pattern of apparent targeting of SCs to produce CSC overabundance is emerging for developmental arsenic exposure that may hold true for the kidney.

Previous studies have shown that many cancer cell hallmarks³⁴ are acquired by cells during *in vitro* chronic exposure to environmentally relevant levels of arsenic.^{13,15,24,25} Often elevated in cancerous cells, matrix metalloproteinases (MMPs) are extracellular matrix-degrading enzymes that have multiple functions in the tumor microenvironment and the carcinogenic process, including the promotion of cell growth, cell survival, invasion, and metastasis.²³ The increases in MMP-9 and MMP-2 seen in the current study are consistent with our previous studies of arsenic-induced malignant transformation,^{24,25} including direct transformation of prostate SCs.¹³ Arsenic exposure also increased the invasive ability of the RIMM-18 cells. Invasion is a key capability for cancer cells that allows for extension, dissemination, and formation of secondary tumors at distant sites (i.e., metastases), an outcome that is lethal in most cancers. The ability of cells to grow in an anchorage-independent manner is also a common characteristic of cancer cells, and in general, the ability of cancer cells to form colonies in soft agar is positively correlated with their *in vivo* tumorigenicity.²⁷ The early, sustained increase in colony formation in arsenic-exposed RIMM-18 cells is consistent with cancer cell characteristics. The increase in sphere forming capacity and in overall sphere size, and the rapid formation of large, highly branched aggressive-appearing ductal-like structures by these spheres is consistent with previous studies of arsenite-induced malignant transformation of human SCs.¹³ Together, these data suggest an increase in self-renewal capacity and an aggressive phenotype in the arsenite-treated SCs consistent with CSCs. Minimally, these multiple cancer-relevant alterations in arsenic-exposed kidney SCs are consistent with multiple acquired cancer cell characteristics.

Cyclooxygenase-2 (Cox-2) is overexpressed in a variety of cancers, including kidney cancer,^{28,29} and is a key mediator of several of the hallmarks of cancer, including evasion of apoptosis, increased proliferation, and increased tissue invasion and metastasis.³⁵ Moreover, Cox-2 expression is induced by arsenic exposure^{36,37} and is overexpressed in arsenic-induced cancers.^{33,38} The anticancer effects of selective Cox-2 inhibitors (i.e NSAIDs) further support the important role that Cox-2 plays in tumorigenesis.²⁸ On the basis of these roles of Cox-2 in carcinogenesis and the fact that Cox-2 expression is commonly up-regulated in arsenic-exposed cells *in vitro* and arsenic-induced tumors *in vivo*, the increase seen in the current study likely offers further, genetic evidence of the transformation of these SCs.

Wnt signaling plays a fundamental role in tissue homeostasis and kidney development.^{20,39} In this regard, *Wnt4* is expressed in kidney SCs and is required for normal development of the metanephric kidney.^{40,41} Dysregulation of the Wnt pathway plays a key role in the formation of cancer SCs and kidney cancer (i.e., Wilms' tumor).^{39,42} In the present study, during arsenic exposure, *Wnt4* expression was first decreased but then up-regulated to a level above that of untreated control cells. The reactivation of *Wnt4* could be due to the increase in *Cox-2* (see above).⁴³ A similar "U-shaped" trend in the expression of SC-related markers has previously been observed during human prostatic SC transformation directly with arsenic,¹³ indirectly via signaling factors released from arsenic-transformed malignant prostate epithelial cells,⁴⁴ and during the progression of hematopoietic SCs to leukemic SCs.⁴⁵ Wnt proteins can activate their signaling in both β -catenin-dependent (canonical pathway) and β -catenin-independent (noncanonical pathway) fashions.³⁹ The absence of Wnt signaling can lead to β -catenin degradation, and mutations in β -catenin can activate Wnt signaling.⁴⁶ Similar to previous studies of arsenite and SCs,⁴⁷ arsenite exposure repressed β -catenin in these renal SCs. This repression could be the result of the initial decrease in *Wnt4* and an increase in Wilms' tumor 1 protein (*Wt1*), a negative regulator of β -catenin signaling.⁴⁸ The decrease in β -catenin may also be due to an arsenic-induced mutation in this gene or may be a way for the developing cancer cells to obtain the level of β -catenin optimal for tumor formation.⁴⁹ Whatever the reason, the decrease in β -catenin may play a role in the reactivation of *Wnt4*. These possibilities require further investigation.

Wilms' tumor protein 1 (*Wt1*) is essential for normal kidney development and is expressed in the undifferentiated metanephric mesenchyme and in the embryonal-type renal cancer known as Wilms' tumors.²⁰ In the kidney, *Wt1* can have dual functionality, acting as both a tumor suppressor gene and as an oncogene depending on differentiation status.⁵⁰ The inactivation of *Wt1* in Wilms' tumors is an early genetic event that leads to kidney SC immortalization.⁵¹ The early inactivation of *Wt1* seen in the present study may be an effect of arsenic down-regulating the tumor suppressor function that causes an overabundance of these renal SCs and thereby expands the metalloid's prospective target cells, as seen in other models with arsenic.^{12–15} The subsequent reactivation of *Wt1* may result from *Wt1* taking on an oncogenic function and maintaining these cells in a distorted CSC-like stage of differentiation.

Arsenic exposure caused a sustained decrease in bone morphogenic protein 7 (*Bmp7*) in these renal SCs, possibly caused by the increase in Cox-2 expression.⁴³ *Bmp7* is necessary for kidney development, prevents renal injury, and stimulates repair in the kidney.^{52,53} Decreased *Bmp* signaling has been shown to enhance Wnt activity and SC self-renewal and suppress SC differentiation,^{43,54} which could possibly explain the reactivation of *Wnt4* seen during arsenic exposure in the RIMM-18 cells and may also be a mechanism by which arsenic maintains a SC population for transformation.^{14,55} This apparent expansion of SC numbers during arsenic transformation is supported by the increased expression of several renal SC markers in the arsenite-exposed RIMM-18 cells in this study, such as *Wt1* and

Wnt4 (described above), Ncam, Osr1, CD24, and CD133. Ncam is a surface marker often used to identify both normal and malignant renal SCs, including the SC fraction found in Wilms' tumors.^{56,57} Osr1, a transcription factor whose expression is confined to undifferentiated kidney precursor tissue, is proposed to maintain renal SCs in an undifferentiated state.⁵⁸ CD24 and CD133 are cell surface markers whose expression is characteristic of the renal SCs in the developing kidney in rodents and humans as well as in renal CSCs.^{49–61} The marked increases in these markers seen in this study suggest that arsenic can modify rodent kidney SCs *in vitro* to acquire cancer characteristics and impact renal cancer development such that CSCs appear to be more frequently produced within aggressive renal cancers, at least compared to ENU.

In this study, arsenic appears to affect several important signaling pathways including Wnt/ β -catenin, Cox-2, and Bmp, during the transformation of renal SCs. The possible cross-talk between pathways may be necessary since an alteration in just one of these pathways is not always sufficient for the development of cancers.⁴³ Furthermore, Wnt signaling has been implicated in Wilms' tumor, where Wt-1 and β -catenin mutations are often found.²⁰ Consistent with previous studies where arsenic induced transformation of SCs into CSCs either directly or indirectly,^{13,44} several SC-related factors in the RIMM-18 cells show a U-shaped trend in expression during the arsenic-induced transformation process. It is interesting to note that most of these factors have dual roles, i.e., they (1) play key roles in development and SC regulation/maintenance and in CSC and cancer formation (i.e., Wnt, Notch, and Wt1) and/or (2) can act as a tumor suppressor and as an oncogene (i.e., Notch and Wt1). This intriguing observation demonstrates that arsenic-induced transformation is a dynamic process and that the expression of some factors during this process may depend on the stage of differentiation of the cells and the stage of carcinogenesis. This has been suggested previously for Wt-1 expression in kidney cancer development.⁵⁰

Previous studies have shown that many types of cells adapt during chronic exposure to inorganic arsenic.^{13,61} This adaptation appears to increase cell survival but can actually facilitate arsenic-induced carcinogenesis by distorting glutathione and methyl metabolism.⁶¹ Most of the adaptation factors examined in this study showed only modest increases before the acquisition of cancer phenotype in the RIMM-18 cells. Mt-2 was the only adaptation factor to show early increases concurrent with phenotypic changes. The reason for this trend in expression levels and their relationship with acquired cancer phenotype in the current study require further investigation.

The transformation of kidney SCs seen in this study is consistent with arsenic-induced direct or indirect SC transformation in other cells.^{13,15,28,44} Coupled with multiple recent reports of arsenic-induced early life exposures causing cancer later in life in humans⁴ and rodents,^{5,6,9,36} this study provides further evidence that, at least in some tissues, arsenic may target SCs for carcinogenic transformation. The targeting of SCs during early stages of life may be key for the development of arsenic-induced cancers in some tissues, including the kidney.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. Y. Xu and O. Ngalame for their critical comments and Mr. Matt Bell for assistance with figure preparation. We thank Dr. Bhal A. Diwan for his assistance with immunohistochemistry.

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ABBREVIATIONS

Bmp	bone morphogenic protein
COX-2	cyclooxygenase-2
E2	estradiol
FBS	fetal bovine serum
FGF	fibroblast growth factor
INT	<i>p</i> -iodonitrotetrazolium
MMP	matrix metal-loproteinase
MT	metallothionein
Ncam	neural cell adhesion molecule
NSAID	nonsteroidal anti-inflammatory drug
Osr1	odd-skipped related 1
PC	progenitor cell
RCC	renal cell carcinoma
SC	stem cell
WT	Wilms' tumor
Wt1	Wilms' tumor protein 1

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Figure 1.

Lethal concentration in 50% of cells (LC₅₀) after inorganic arsenic exposure. RIMM-18 cells were treated with indicated concentrations of arsenite for 72 h to determine cell survival. The LC₅₀ value for arsenite-treated cells was derived from the survival curve. Data are given as the mean LC₅₀ values $\pm$ standard deviation ($n = 6$). Asterisks (*) indicate a significant difference ($p < 0.05$) from the control.

Figure 2.

In vitro metrics of acquired cancer phenotype in chronic arsenite-treated (500 nM) RIMM-18 kidney stem/progenitor cells. (A) Secreted MMP-9 and MMP-2 activity over 28 weeks of arsenite exposure. (B) Colony forming efficiency and (C) invasion during 28 weeks of arsenite exposure. (D) Cyclooxygenase-2 (*Cox-2*) mRNA levels at 28 weeks of chronic arsenite exposure. (E) Number of (bottom) and representative size of (top) nonadherent free-floating spheroid bodies produced by control and arsenite-treated (28 weeks) kidney SCs. Bar = 100 μ m. (F) Ductal-like structures formed in Matrigel from spheroid cells derived from arsenite-exposed cells (bottom) show an aggressive phenotype indicative of CSCs when compared with control spheres (top). Bar = 50 μ m. Data represent the mean $\pm$ standard deviation ($n = 3$). Asterisks (*) indicate significant difference compared with control cells; $p < 0.05$.

Figure 3.

Expression of genes associated with kidney development and/or kidney SCs during chronic arsenite exposure in RIMM-18 kidney SC/PCs. Transcriptional expression (mRNA) normalized to β -actin and *Gapdh* and set to control = 100%. (A) *Wnt4*; (B) β -catenin; (C) *Bmp-7*; and (D) *Wt-1*. Asterisks (*) indicate significant differences compared with the control cells; $p < 0.05$.

Figure 4.

Expression of SC markers after 28 weeks of chronic arsenite exposure *in vitro* in RIMM-18 cells together with evidence of CSC overabundance in arsenical-induced mouse RCC. (A) CD24 mRNA levels in RIMM-18 cells. (B) *Osr1* mRNA levels in RIMM-18 cells. (C) *Ncam* mRNA levels in RIMM-18 cells. (D) *Cd133* mRNA levels in RIMM-18 cells. Asterisks (*) indicate significant differences compared with the control cells; $p < 0.05$. (E) Immunohistological sections showing expression of CD133, a renal CSC marker, in (top) a transplacental arsenical-induced RCC and (bottom) a transplacental ENU-induced RCC. Note the more intense and widespread staining (brown color) for the CD133 protein in the arsenical-induced tumor and numerous cells (examples at arrows) with very intense staining. Arrows in the ENU slide indicate a cell with relatively more pronounced CD133 staining.

Figure 5. Transcript levels of adaptation and oxidative stress related genes during chronic arsenite exposure. (A) *Mt-1*; (B) *Mt-2*; (C) *Sod-1*; and (D) *Abcc2*. Note the difference in y-axes between graphs. Asterisks (*) indicate significant increase compared with the control cells; $p < 0.05$.