Research Article

Arsenic Exposure *In utero* Exacerbates Skin Cancer Response in Adulthood with Contemporaneous Distortion of Tumor Stem Cell Dynamics

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Abstract

Arsenic is a carcinogen with transplacental activity that can affect human skin stem cell population dynamics *in vitro* by blocking exit into differentiation pathways. Keratinocyte stem cells (KSC) are probably a key target in skin carcinogenesis. Thus, we tested the effects of fetal arsenic exposure in Tg.AC mice, a strain sensitive to skin carcinogenesis via activation of the *v-Ha-ras* transgene likely in KSCs. After fetal arsenic treatment, offspring received topical 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) through adulthood. Arsenic alone had no effect, whereas TPA alone induced papillomas and squamous cell carcinomas (SCC). However, fetal arsenic treatment before TPA increased SCC multiplicity 3-fold more than TPA alone, and these SCCs were much more aggressive (invasive, etc.). Tumor *v-Ha-ras* levels were 3-fold higher with arsenic plus TPA than TPA alone, and *v-Ha-ras* was overexpressed early on in arsenic-treated fetal skin. *CD34*, considered a marker for both KSCs and skin cancer stem cells, and *Rac1*, a key gene stimulating KSC self-renewal, were greatly increased in tumors produced by arsenic plus TPA exposure versus TPA alone, and both were elevated in arsenic-treated fetal skin. Greatly increased numbers of CD34-positive probable cancer stem cells and marked overexpression of RAC1 protein occurred in tumors induced by arsenic plus TPA compared with TPA alone. Thus, fetal arsenic exposure, although by itself oncogenically inactive in skin, facilitated cancer response in association with distorted skin tumor stem cell signaling and population dynamics, implicating stem cells as a target of arsenic in the fetal basis of skin cancer in adulthood. [Cancer Res 2008;68(20):8278–85]

Introduction

Compelling evidence indicates that events *in utero* could affect the development of human disease during adulthood (1–3). This concept first arose from data correlating low birth weight with adult-onset disease, indicating that early-life undernutrition caused permanent changes precipitating untoward effects much later in life (1, 2). Fetal chemical exposures similarly could cause adverse effects in adulthood, exemplified by the observation that transplacental carcinogens can initiate oncogenesis *in utero*, thereby producing cancers in adult humans or rodents (4). The time difference between fetal exposure and adulthood cancer requires the underlying etiology to be quiescent for extended periods, but still clearly sensitive to subsequent stimulation. Emerging theories predict that many cancers likely arise in pluripotent stem cell populations (5–7), and the normal stem cell qualities of quiescence, self-renewal, and "immortality" would allow for a lifelong, latent neoplastic cell population (7). Fetal stem cells are probably key targets in transplacental carcinogenesis (4) and may affect fetal sensitivity based, at least in part, on relative abundance. However, the identity of the normal cells that first acquire the molecular lesions initiating cancer remains elusive (8), regardless of the life-stage of initiation.

Inorganic arsenic is a transplacental carcinogen in mice (9) and probably humans (10). Fetal exposure to arsenic in mice can induce or initiate tumors or preneoplasias in a wide variety of tissues in adulthood (9), including several concordant with human sites of arsenic carcinogenesis, like the bladder and lung (9, 11). The propensity for arsenic to act as a multisite transplacental carcinogen seems to be in keeping with fetal stem cells as a critical target population in transplacental carcinogenesis (4). However, skin cancers, although common in arsenic-exposed humans (11), have not been seen after *in utero* arsenic exposure in mice, although the strains used to date are not particularly prone to skin cancer (9). In adult rodents, inorganic arsenic could clearly affect skin carcinogenesis, acting as a cocarcinogen which, by itself, is inactive and only affects tumor development when given with some additional treatment such as topical 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) or UV irradiation (12–14). Recent studies in human skin cancer models *in vitro* indicate that arsenic can slow epidermal stem cell exit into differentiation pathways, thereby distorting population dynamics (15, 16). In effect, arsenic increases the relative proportion of the epidermal stem cells in culture, leading to the hypothesis that this may be a key target cell population for *in vivo* carcinogenesis (15, 16). Accepting that cancer is a disease driven by stem/progenitor cell dysregulation (5), an arsenic-induced increase in skin stem cell number or similar dysregulation *in vivo* might potentially remain quiescent yet facilitate cancer after subsequent stimulation, although this has not been tested directly.

Based on converging hypotheses that arsenic as a transplacental carcinogen may target fetal stem cells and that it may have the ability to affect skin stem cell population number/dynamics, the effects of fetal arsenic exposure on skin carcinogenesis were tested in Tg.AC transgenic mice, a strain sensitive to skin cancer (17–19).

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Tg.AC mice are well-suited for this study as they are susceptible to the development of skin cancers via the activation of the *v-Ha-ras* transgene, probably when the activation occurs in keratinocyte stem cells (KSC; refs. 17, 18). Activation of the transgene in KSCs is very likely a key, early event in Tg.AC skin carcinogenesis (17, 18) and Tg.AC skin is considered genetically preinitiated with KSCs representing a latent neoplastic stem cell population needing only appropriate stimulation to develop tumors (17). These mice typically develop papillomas, and to a lesser extent, squamous cell carcinoma (SCC; ref. 17). SCC is common in humans in which arsenicosis is endemic (11) and in adult mouse skin cancer models using arsenic with other skin carcinogens (14). A further advantage to this model system is that a specific molecular marker for KSCs in mouse skin, i.e., *CD34*, has been well-defined in Tg.AC mice (17–19). *CD34* expression not only seems critical to the carcinogenic process in mouse skin (18), but has now also been convincingly established as a marker for cancer stem cells (CSC) in murine epidermal skin tumors like SCC (20).

Materials and Methods

Animals and treatment. Animal care was provided in accordance with the U.S. Public Health Policy on the Care and Use of Animals as defined in the Guide to the Care and Use of Animals (NIH Publication 86-23). Mice were housed under conditions of controlled temperature, humidity, and light cycle. The tumor study was performed at the National Cancer Institute-Frederick animal facility (Frederick, MD). Fetal skin sample collection was performed at Integrated Laboratory Systems, Inc. (Research Triangle Park, NC). Homozygous Tg.AC mice were obtained from Taconic Farms and used in both studies.

For skin tumors, 30 timed primigravid mice were randomly divided into groups of 10 and given drinking water with sodium arsenite ($NaAsO_2$) at 0 (control), 42.5 ppm arsenite (low), or 85 ppm arsenite (high) *ad libitum* from days 8 to 18 of gestation. After birth, offspring were weaned at 4 weeks and randomly put into groups (initial n = 50) of equal numbers of males and females based on maternal exposure and then exposed to TPA (2 μg/0.1 mL acetone, twice a week to a shaved area of the dorsal skin) or vehicle (acetone) for 36 weeks. Dam body weights and water consumption were recorded during gestation. Offspring weights were recorded weekly from birth. Mice were checked daily and sacrificed when significant clinical signs developed or at 40 weeks.

To assess molecular changes in fetal skin, timed primigravid mice were given drinking water containing sodium arsenite at 0 or 85 ppm arsenite *ad libitum* from days 8 to 18 of gestation (five mice/group). On gestation day 19, dams and fetuses were sacrificed and samples of fetal skin were taken for analysis.

Tumor assessment. During TPA exposure, tumor growth was monitored carefully. Mice found moribund or cachetic or with a tumor the size of 1.5 × 1.5 cm were euthanized to alleviate pain or distress. Remaining mice were killed at 40 weeks. Skin tumors were fixed in buffered formalin, sectioned and stained with H&E. All skin tumors were assessed histologically, and pathologic stage and number per animal were determined. Some tumors were also used for immunochemistry or molecular analysis. A complete necropsy was also performed and non–skin lesions that occurred will be reported elsewhere.

Tumor immunohistochemistry. Skin tumors were compared for localization and intensity of RAC1 and CD34. For this, advanced papillomas with clear mesenchymal and epithelial components and monoclonal anti-RAC1 and anti-CD34 antibodies (BD Biosciences) and Vector Elite kits (Vector Labs) were used. The primary antibodies were used at a 1:100 (CD34) or 1:10,000 (RAC1) dilutions. To define specificity, the primary antibodies were omitted from each staining series as a control.

Gene transcript analysis. Total RNA was isolated with TRIzol agent (Invitrogen), and purified (RNeasy columns, Qiagen), and transcript levels were quantified using real-time reverse transcription-PCR analysis. Total RNA was reverse-transcribed with High-Capacity cDNA reverse transcription kit (Applied Biosystems), and subjected to real-time-PCR analysis using either Power SYBR Green PCR Master Mix (*Rac1, CD34, DSS1, cyclin D1, Sprr2a, Ptges*, and *Colla2*) or TaqMan Universal PCR Master Mix (*v-Ha-ras* and *p16*; Applied Biosystems). Cycle time (Ct) values were normalized with 18S from the same sample, and the relative differences were calculated and expressed as a ratio of control (set as 1.0) or as a percentage of TPA alone (set as 100%). For consistency, only males were used for transcript analysis.

Data analysis. Data are expressed as mean ± SE or as a percentage of mice at risk (incidence). Papilloma and SCC multiplicity represents mean tumors/mouse and comes from 47 to 49 mice per treatment group originally derived at random from 10 litters per treatment group. Papillomas in TPA-treated mice also progressed to mesenchymal spindle cell sarcomas (21) but as fetal arsenic had no effect on these tumors, they were not considered further. Adult transcript data were from four to five individual samples randomly selected from control nontumor skin, or papilloma and SCC from the TPA alone, or 85 ppm arsenic plus TPA groups. Fetal skin transcript data were from 10 to 12 individual samples randomly selected from the control or the arsenic group from five litters per treatment group. Tumor multiplicity and transcript levels were tested by ANOVA and Dunnett's test against control or by Tukey-Kramer multiple comparisons test for cross-comparing treated groups. Because initial analysis of tumors showed that no gender-based differences occurred between similarly

Table 1. Fetal arsenic exposure increases malignant skin cancer susceptibility in Tg.AC mice

Treatment group	n	Papilloma multiplicity (papilloma/mouse)	SCC multiplicity (SCC/mouse)	Mice with three or more SCC/total mice (%)
Control	49	0.45 ± 0.17	0.04 ± 0.02	0/49 (0%)
Low arsenic	47	0.94 ± 0.31	0.06 ± 0.04	0/47 (0%)
High arsenic	48	0.12 ± 0.05	0.04 ± 0.03	0/48 (0%)
TPA alone	47	17.0 ± 1.38*	0.57 ± 0.11*	1/47 (2.1%)
Low arsenic plus TPA	48	16.9 ± 1.40*	1.31 ± 0.20*,†	9/48 (18.8%)*,†
High arsenic plus TPA	49	11.1 ± 1.22*,†	1.49 ± 0.21*,†	14/49 (28.6%)*,†

NOTE: Mice were exposed to arsenic *in utero* via maternal drinking water containing either 0, 42.5 ppm (low arsenic), or 85 ppm (high arsenic) of arsenic and given TPA dermally from weaning through adulthood (see Materials and Methods for details). Very robust, arsenic dose-response relationships occurred in mice treated with TPA (TPA alone, Low arsenic plus TPA, and High arsenic plus TPA groups) for SCC multiplicity and for mice with three or more SCC (trend $P < 0.0001$ for both).

* $P < 0.05$, significant difference from control.

† $P < 0.05$, significant difference from TPA alone.

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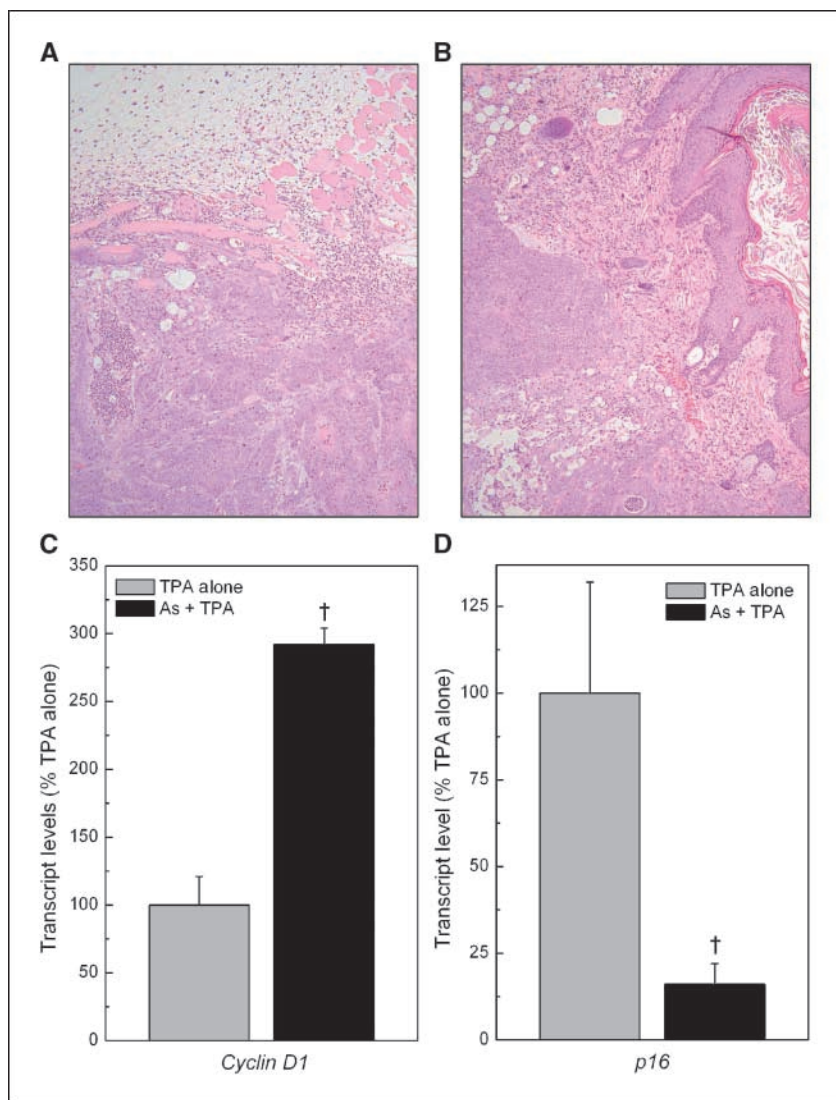

Figure 1. Fetal arsenic exposure enhances skin cancer aggressiveness. SCC induced by fetal arsenic exposure and subsequent dermal TPA during adulthood often showed areas of local invasion into the subdermal muscle and infiltration of the dermis. *A,* a SCC induced by fetal arsenic exposure and subsequent dermal TPA during adulthood clearly showing invasion into regional muscle (original magnification, ×100). *B,* a SCC induced by fetal arsenic exposure and subsequent dermal TPA during adulthood clearly showing infiltration (invasion) into the dermis (original magnification, ×100). SCC induced by fetal arsenic exposure and TPA during adulthood commonly showed signs of rapid proliferation, such as frequent mitotic figures (data not shown). All histopathology was assessed after staining with H&E. *C,* transcriptional expression of *cyclin D1* in SCC induced by fetal arsenic exposure and subsequent dermal TPA during adulthood (*As + TPA*) or by TPA alone. †, $P < 0.05$, significant difference from TPA alone. *D,* transcriptional expression of *p16* in SCC induced by fetal arsenic exposure and subsequent dermal TPA during adulthood (*As + TPA*) or by TPA alone. †, $P < 0.05$, significant difference from TPA alone.

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treated groups of males and females, they were pooled for final assessment. Incidence data (mice with three or more SCC) were compared by Fischer's exact test. Arsenic dose-response relationships across TPA-treated groups (TPA alone, low arsenic plus TPA, and high arsenic plus TPA) were determined by correlative trend tests for SCC multiplicity (Pearson correlation) or for incidence of mice with three or more SCC (χ^2 test for trend). $P \leq 0.05$ was considered significant in all cases.

Results and Discussion

Fetal arsenic exposure, skin cancer susceptibility, and tumor aggressiveness. Pregnant Tg.AC mice received arsenic in the drinking water from gestation days 8 to 18 at doses that resulted in fetal blood inorganic arsenic levels similar to humans with arsenicosis and that induced non–skin tumors in the offspring of other mouse strains (9). The doses were well tolerated by the dam, fetus, and neonate based on maternal water consumption, maternal and neonatal body weights, and weight gains of the offspring (data not shown). Although these external doses are higher than what are typically encountered by humans in the environment, they are nontoxic to the mice and apparently because mice differ from humans in much more rapid clearance of arsenic, they lead to a similar internal target tissue dosimetry (9). After birth and weaning, the offspring received topical TPA through adulthood to 40 weeks of age. Controls or mice given arsenic alone developed few skin tumors whereas TPA alone induced papillomas and SCC (Table 1). Papilloma multiplicity (tumors/mouse) in TPA-treated mice was either not altered or modestly reduced by prior fetal arsenic exposure. However, SCC multiplicity and the incidence of mice with three or more SCC were markedly increased with fetal arsenic treatment prior to TPA in adulthood when compared with TPA alone (Table 1). Both SCC multiplicity and mice with three or more SCC showed highly significant arsenic dose-relationships in TPA-treated mice (via correlative trend tests; see Table 1).

Not only were malignant tumors more frequent after fetal arsenic prior to TPA but many showed pathologic and molecular indications of being more aggressive. SCC induced by arsenic plus TPA exhibited more frequent local muscle invasion (Fig. 1*A*), infiltration of the dermis (Fig. 1*B*), and more frequent mitotic figures (data not shown). Compared with SCC induced by TPA alone, SCC induced by fetal arsenic plus TPA also showed more aggressive tendencies at the molecular level, reflected in marked

increases in expression of the cell proliferation gene, *cyclin D1* (Fig. 1*C*) and decreases in expression of the tumor suppressor gene, *p16* (Fig. 1*D*).

In Tg.AC mice, all SCC arise from pre-existing papillomas (22), and the larger, aggressive malignancies could incorporate/obliterate the smaller benign lesions, potentially accounting for fewer papillomas with the higher arsenic dose (see Table 1). Both papillomas in mice and early lesions in arsenic-exposed human skin involving hyperkeratosis/nodular keratosis are likely a stage leading to more advanced epidermal proliferative lesions (11). Indeed, evidence indicates that chemically induced papillomas can be either of low or high risk for conversion to SCC (23, 24), and tumors at high risk for conversion arise from carcinogenic "hits" within the epidermal stem cell compartment of the hair follicle (24), likely a key locale for initial *v-Ha-ras* transgene expression in Tg.AC skin carcinogenesis (17, 18). Thus, the increase in malignant conversion to SCC in TPA-treated mice exposed to arsenic *in utero* supports a stem cell origin for these malignancies, and could also be responsible for a loss of papillomas. Overall, fetal arsenic exposure markedly increased high-grade malignancy response in adult Tg.AC mouse skin, consistent with models that point towards a stem cell origin for malignant skin tumors (25).

Tumor expression of *v-Ha-ras*. Tg.AC mouse studies indicate that the levels of *v-Ha-ras* transgene expression dictate carcinogenic response, that KSCs are a latent neoplastic stem cell population, and that transgene activation in KSCs is likely a critical event in the oncogenic process (17–19). Based on the hypothesis that fetal arsenic exposure may affect subsequent stem cell response, *v-Ha-ras* expression was assessed. The *v-Ha-ras* transcript levels in SCC induced by TPA treatment alone were ~4.5-fold higher than control skin but were >14-fold above controls in SCC resulting from fetal arsenic plus TPA (Fig. 2*A*). Expression of *v-Ha-ras* in both papillomas and SCCs was approximately three times higher after fetal arsenic prior to TPA exposure compared with TPA alone (Fig. 2*B*). Thus, fetal arsenic with subsequent TPA caused more SCC and a hyperexpression of *v-Ha-ras* to similar levels in both benign and malignant skin tumors. Because *v-Ha-ras* transgene activation in skin stem cells is very likely a critical event in Tg.AC skin carcinogenesis (17–19) its marked overexpression in tumors induced by arsenic plus TPA indicates that it contributed to increased tumor response.

Expression of the *v-Ha-ras* transgene is increased by promoter region methylation loss (26), and arsenic can activate gene expression via DNA hypomethylation (27). However, fetal arsenic prior to TPA exposure did not alter levels of transgene promoter methylation compared with TPA alone (data not shown). Given the marked increases in *v-Ha-ras* transcript, this argues for more cells overexpressing the transgene as opposed to a few cells expressing the gene at much higher levels, and together with the marked increases in SCC multiplicity, is consistent with an increased target cell hypothesis. Interestingly, it is typical for arsenicosis victims to have a great number of skin lesions, from hyperplasia to tumors, with SCC a common malignancy (11).

Tumor expression of *CD34* and *Rac1* transcripts. Because arsenic *in vitro* affects human epidermal stem cell population dynamics by stalling exit into differentiation (15, 16), the expression of several genes linked to KSC population dynamics were studied. The expression of the specific KSC marker *CD34* (18, 19) was markedly elevated in both papillomas and SCC induced by fetal arsenic plus TPA exposure as compared with TPA alone (Fig. 3*A* and *B*). Relative to control skin, the levels of *CD34* transcript in SCC induced by TPA alone were unchanged but were increased >3-fold in SCC resulting from fetal arsenic exposure plus TPA treatment (Fig. 3*C*). The overexpression of *CD34* in tumors induced by fetal arsenic plus TPA treatment in adulthood is noteworthy because *CD34* is a specific marker for KSCs (19), and its expression is crucial for carcinogenesis in the mouse skin (17, 18), pointing towards KSC as an essential target in skin carcinogenesis (18). More importantly, very recent work provides compelling evidence that CD34-positive tumor cells are, in fact, CSCs for murine epidermal skin tumors, based on their stunning potency for the initiation of secondary tumor formation together with various other stem cell characteristics (20). Thus, these data indicate that fetal arsenic exposure modifies stem cell response to carcinogen exposure in adulthood, potentially resulting in more CSCs, such that more carcinomas of higher aggressiveness occur.

The transcript levels of *Rac1*, a key gene in skin stem cell population dynamics which both stimulates KSC self-renewal and

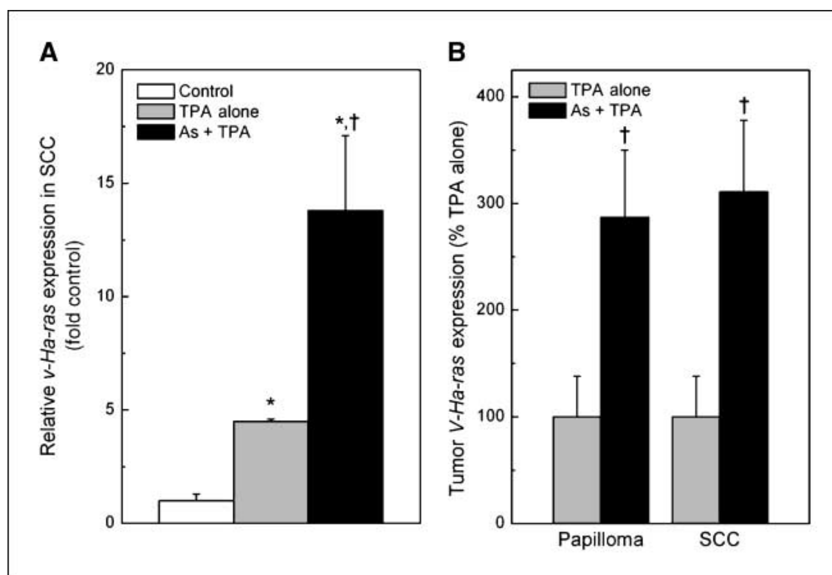

Figure 2. Effect of fetal arsenic exposure on tumor *v-Ha-ras* transgene transcriptional expression. *A,* relative expression of the *v-Ha-ras* in SCC induced by fetal arsenic exposure and subsequent dermal TPA during adulthood (*As + TPA*) or by TPA alone as compared with control nontumor skin. *B,* expression of the *v-Ha-ras* transgene cross-compared between TPA alone and arsenic plus TPA (*As + TPA*) in papillomas and SCCs. *, $P < 0.05$, significant difference from control; †, $P < 0.05$, significant difference from TPA alone.

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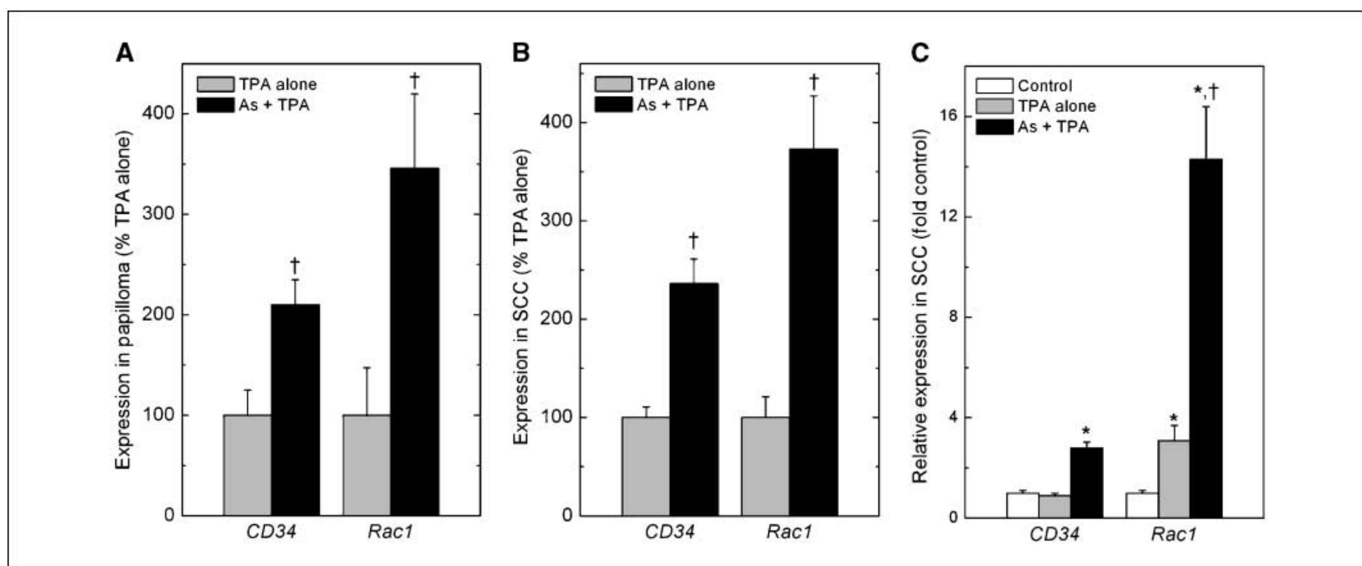

Figure 3. Tumor expression of *CD34* and *Rac1* transcript. *A,* expression of *CD34* and *Rac1* in papilloma induced by fetal arsenic exposure and subsequent dermal TPA during adulthood (*As + TPA*) compared with papilloma induced by TPA alone. *B,* expression of *CD34* and *Rac1* in SCC induced by fetal arsenic exposure and subsequent dermal TPA during adulthood (*As + TPA*) compared with SCC induced by TPA alone. *C,* relative expression of *CD34* and *Rac1* transcripts in SCC induced by fetal arsenic exposure and subsequent dermal TPA during adulthood (*As + TPA*) or by TPA alone as compared with control nontumor skin. *, $P < 0.05$, significant difference from control; †, $P < 0.05$, significant difference from TPA alone.

suppresses transient-amplifying cell (TAC) production (28), were increased >3.5-fold by prenatal arsenic treatment before TPA exposure in both papilloma and SCC (Fig. 3*A* and *B*). Relative to control skin, *Rac1* transcript levels in SCC induced by TPA treatment alone were elevated 300% but were >1,400% higher in SCC resulting from prenatal arsenic exposure plus TPA treatment in adulthood (Fig. 3*C*). Prior work indicates that *Rac1* is overexpressed in SCC (28), and its expression seems to be key to the maintenance of a malignant phenotype in SCC cells *in vitro* (29). Consequently, it has been hypothesized that *Rac1* overexpression should induce skin hyperproliferation and, potentially, skin cancers (28). In accord with this hypothesis, the remarkable overexpression of *Rac1* facilitated by prior fetal arsenic exposure greatly increased chemically induced SCC formation in adulthood. Overall, these data provides evidence of more skin stem cells (increased *CD34*; refs. 17–20) and of signaling potentially increasing stem cell production (increased *Rac1*; ref. 28).

The expression *c-myc*, which normally controls the production of TACs under the control of *Rac1* in the skin (28, 30), in SCC was unchanged by TPA treatment regardless of prenatal arsenic exposure (TPA alone, 100 ± 20%; arsenic plus TPA, 84 ± 19%). Thus, fetal arsenic exposure enhances tumor *Rac1* expression, potentially signaling increased production of stem cells, but the molecular signal (*c-myc*) dictating progression into TACs and on to terminal differentiation (28, 30) is unaltered. This is consistent with arsenic *in vitro* stalling exit of human epidermal stem cells into differentiation pathways and supports a hypothesis that arsenic may increase the number and/or persistence of target cells for skin cancer initiators or promoters (15, 16).

Tumor expression of CD34 and RAC1 protein. Immunohistologic analysis strongly supports the contention that prenatal arsenic distorted the response to subsequent TPA exposure, including stem cell response. In this regard, advanced papillomas with clear mesenchymal and epithelial components were assessed for CD34-positive stem cells and RAC1 protein expression. CD34-positive cells occurred much more frequently in tumors induced by fetal arsenic prior to TPA (Fig. 4*A* and *B*) compared with tumors from TPA treatment alone (Fig. 4*C*), indicating a remarkable increase in the number of stem cells (KSCs/CSCs; refs. 17–20) after fetal arsenic. CD34 occurred intensely in the cells of the epithelial component of the tumor. Few CD34-positive cells were seen in tumors induced by TPA alone, which would be expected for a tumor with a proportionately small stem cell population. Given the compelling recent evidence that CD34 is a clear marker of CSCs in mouse epidermal skin tumors (20), it therefore seems that prior fetal arsenic exposure greatly increases skin tumor CSCs. SCCs induced by arsenic plus TPA also often showed intensely positive CD34-containing cells at the invading front (data not shown), in keeping with recent data identifying CSCs as a primary source of tumor invasion (20) and consistent with the more aggressive nature of the SCC from this treatment group (see Table 1). As expected, hair follicle bulge regions were positive for CD34 in the surrounding normal skin (Fig. 4*D*) due the presence of KSCs (19).

RAC1, a key component in signaling for skin stem cell replication and renewal as well as in negative control of TAC production (28), was greatly increased in tumors induced by fetal arsenic prior to TPA (Fig. 5*A–C*) compared with tumors induced by TPA alone which showed minimal RAC1 (Fig. 5*D*). With exposure to fetal arsenic plus TPA, RAC1 appeared most intensely on cell membranes. In the liver, arsenic induces sustained signaling involving hepatic endothelial *Rac1* activation that markedly increases membrane-bound levels of RAC1 protein (31). Overall, compared with TPA alone, the tumors induced by fetal arsenic and subsequent TPA showed more CSCs, as recently defined by CD34 positivity (20), and distorted signaling (increased RAC1) that could lead to overproduction of stem cells (28).

Changes in fetal skin potentially relevant to epidermal carcinogenesis. To see if changes potentially relevant to epidermal carcinogenesis occurred as an early event, *CD34, Rac1*, and *v-Ha-ras* transcripts were assessed in fetal skin after arsenic

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exposure on gestation day 19. *CD34*, when compared with control fetal skin (100 ± 11%), was increased after fetal arsenic exposure (196 ± 49%, $P < 0.05$). *Rac1* also increased after fetal arsenic exposure (175 ± 18%, $P < 0.05$) compared with controls (100 ± 11%). Levels of the *v-Ha-ras* were increased by fetal arsenic treatment (252 ± 58%, $P < 0.05$) compared with controls (100 ± 30%). These arsenic-induced changes in fetal skin mimic those seen with arsenic facilitation of skin tumors in adulthood, and support the hypothesis based on *in vitro* work that arsenic acts to quantitatively increase stem cells as a target population as part of its carcinogenic mode of action in skin (15, 16). Increases in *CD34, Rac1*, and *v-Ha-ras* could foreshadow the distortion of stem cell responsiveness and/or numbers contributing to adulthood cancer. Staining for CD34 and Rac1 protein in fetal skin after fetal arsenic proved inconclusive, perhaps because mature structures associated with KSCs in mouse skin, such as hair follicles, are not yet formed at gestation day 19 in mice.

SCC expression of genes specific to *v-Ha-ras*–driven skin cancer. *DSS1* is a TPA-inducible gene expressed in keratinocyte progenitor cells influenced by *v-Ha-ras* that plays an important role in skin carcinogenesis (32). *DSS1* expression was significantly increased in SCC induced in adult skin by arsenic plus TPA (12.0 ± 3.0-fold above control skin) compared with TPA alone (4.1 ± 0.8-fold; $P < 0.05$). *DSS1* expression was increased ~2-fold in papilloma and ~3-fold in SCC induced by fetal arsenic plus TPA compared with TPA alone (data not shown). An early response gene in skin hyperplasia, *DSS1* overexpression is common to TPA-induced tumors, and functional analysis suggests that it is required for epidermal cell proliferation and oncogenic transformation (32). Thus, *DSS1* overexpression after fetal arsenic exposure is consistent with enhanced multiplicity of more aggressive skin cancers.

Other genes for which activation is specific in *v-Ha-ras*–driven skin tumorigenesis in Tg.AC mice (33) were also significantly ($P < 0.05$) increased in SCC induced by arsenic plus TPA including *Sprr2a* (TPA alone, 8.4 ± 1.3-fold over control skin; arsenic plus TPA, 17.7 ± 3.8-fold), *Ptges* (TPA alone, 7.5 ± 1.8-fold; arsenic plus TPA, 18.5 ± 5.3-fold), and *Col1a2* (TPA alone, 2.8 ± 0.8-fold; arsenic plus TPA, 17.2 ± 3.9-fold). Thus, fetal arsenic exposure increased the capacity for *v-Ha-ras* transgene expression resulting in an exaggerated downstream molecular response.

Implications. Fetal arsenic exposure alone did not produce skin tumors in the present study yet clearly increased the carcinogenic response for high-grade malignancies. With a short biological half-life (~4 days; ref. 11), it is unlikely that large amounts of arsenic remain to directly affect oncogenesis in adults following fetal exposure. Thus, fetal arsenic facilitates, but on its own is insufficient for, skin carcinogenesis via a mechanism not requiring its own direct participation. Any mechanistic hypothesis must account for the quiescence of fetal arsenic exposure in skin and yet provide for the remarkable capacity to stimulate activity of other

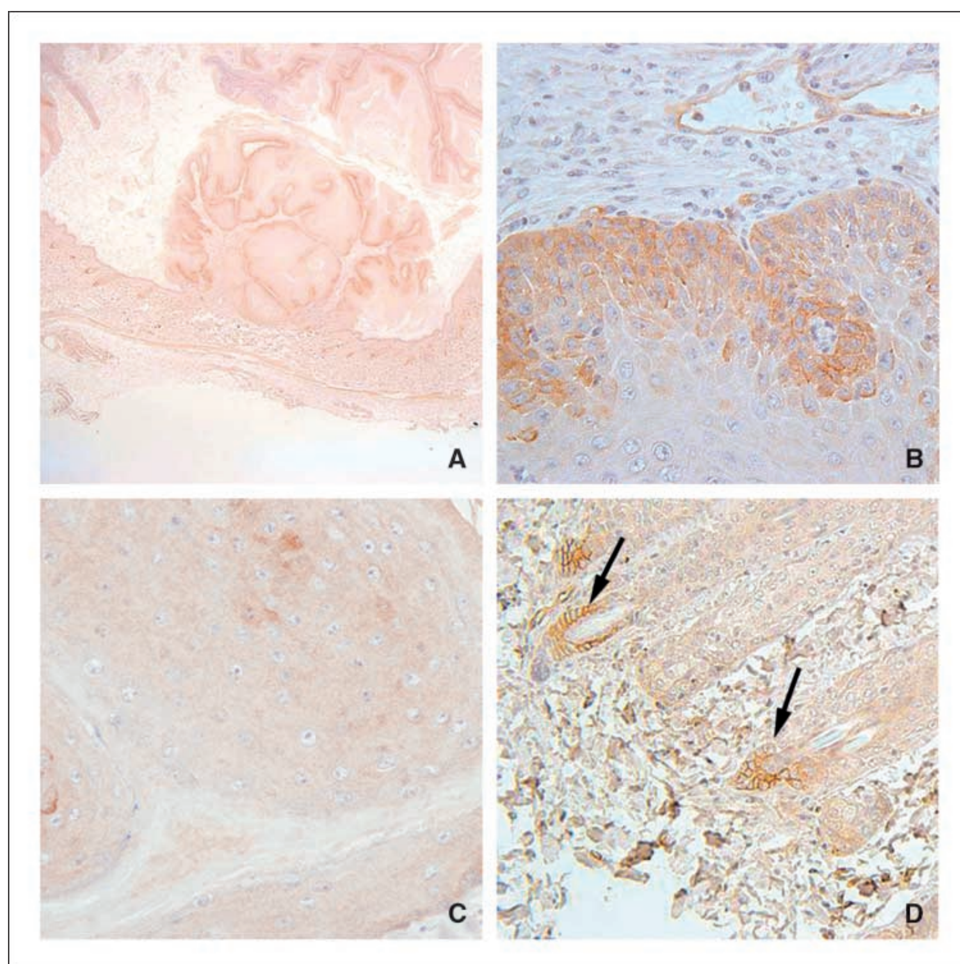

Figure 4. Tumor expression of CD34 protein. *A,* typical tumor induced by fetal arsenic exposure plus TPA treatment in adulthood showing intense and widespread presence of the CSC marker CD34 in its epithelial portion (original magnification, ×20). Positive staining is brown. *B,* a higher magnification of the tumor in *A* showing intense staining for CD34-positive probable CSCs (original magnification, ×200). *C,* a typical tumor induced by TPA alone showing little evidence of CD34 staining (original magnification, ×200). *D,* hair follicle bulge regions (*arrows*) showing positive staining for CD34 which is typical for normal KSCs (original magnification, ×20).

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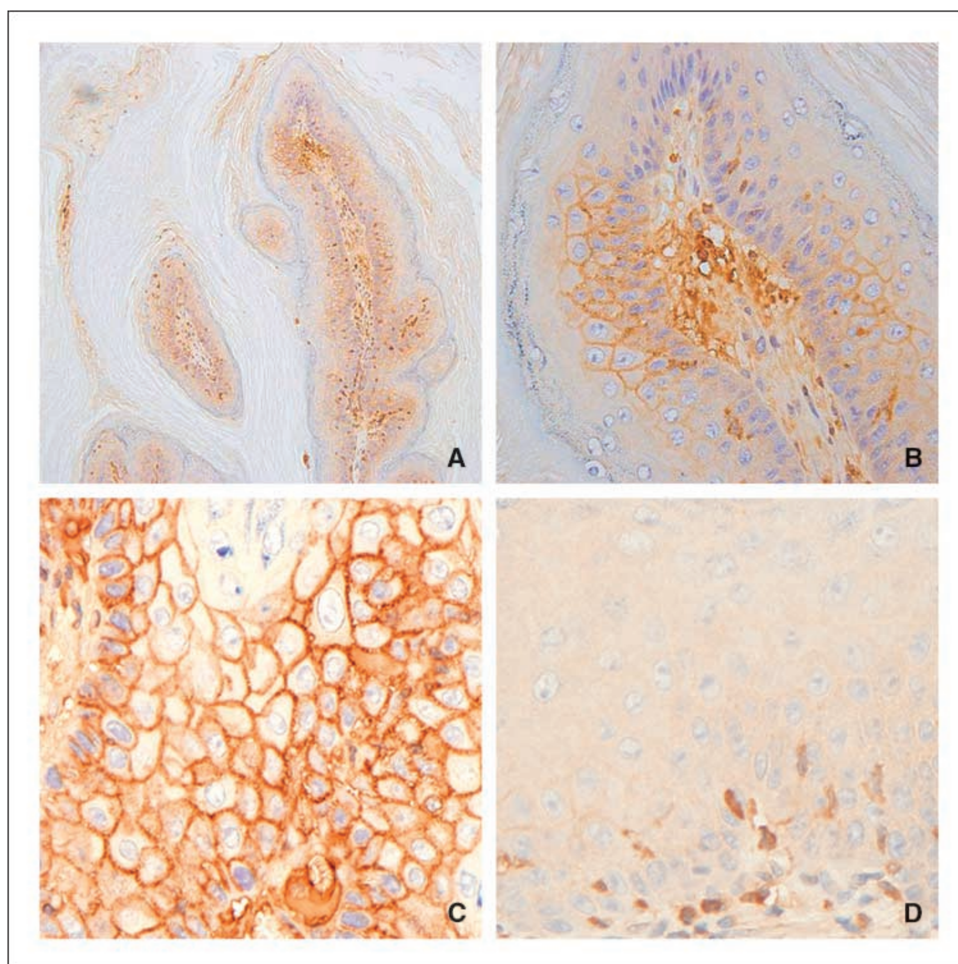

Figure 5. Tumor expression of RAC1 protein. *A,* typical tumor resulting from fetal arsenic exposure plus TPA during adulthood showing intense and widespread presence of RAC1 in the epithelial and stromal portions of the tumor (original magnification, ×20). Positive staining is brown. *B,* a higher magnification of the tumor in *A* with intense staining for RAC1 (original magnification, ×200). *C,* RAC1 staining was pronounced at the cell surface of the epithelial portion of tumors induced by fetal arsenic plus TPA (original magnification, ×400). *D,* RAC1 was largely absent in tumors produced by TPA alone (original magnification, ×400).

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agents given subsequently. In adult mice, although linkage to stem cell dynamics/signaling has not been tested, inorganic arsenic cotreatment increases both TPA- and UV-induced skin tumorigenesis, but is similarly inactive when given alone (12–14). This has led to the concept that arsenic acts as a skin cocarcinogen (14). In this case too, an arsenic-induced effect on adult skin stem population dynamics or numbers might fulfill the mechanistic requirement of arsenic dormancy alone whereas providing the basis for enhancing the activity of other agents. Because it seems that arsenic can quantitatively affect skin stem cells by blocking differentiation pathways *in vitro* (15, 16), similar studies should be performed on skin stem cell dynamics in *in vivo* models of arsenic-induced skin cocarcinogenesis with environmentally relevant agents like UV irradiation. In fact, earlier work with arsenic in adult Tg.AC mice lead to the suspicion that it acted as a skin cocarcinogen/copromoter by stimulating a pluripotent stem cell population (13).

Cancer is increasingly viewed as a stem cell disease, even in its early stages (5, 6, 25, 34). This hypothesis conceptually involves tumor origination from stem/progenitor cells after dysregulation of the normally tightly regulated process of self-renewal and differentiation (5, 6). Furthermore, cancer cells, especially CSCs, retain stem cell–like properties, such as self-renewal, and show differentiation often in a highly distorted fashion (5, 6). Overall, because *Rac1* seems to drive KSC replication and inhibits TAC production (28) and *CD34* is a specific marker for mouse KSCs (18, 19) and skin epidermal CSCs (20), the present data supports the notion that fetal arsenic exposure has quantitatively or qualitatively influenced skin stem/progenitor cells in Tg.AC mice such that a carcinogenic stimulus later in life has more target cells and thereby results in a greatly magnified malignant tumor response including overproduction of CSCs. Accumulating data from various skin cancer models strongly indicate that most tumors with high malignant potential, analogous to the SCC in the present study, originate from the epidermal stem cell population (20, 25). Tumors resulting from fetal arsenic plus TPA in this study retained stem cell–like qualities and showed an increased propensity towards malignancy and a heightened tumor aggressiveness, whereas CD34 is often overexpressed in more advanced and aggressive SCCs (20). In addition, data from fetal skin exposed to arsenic *in utero*, where expression of *CD34, Rac1*, and *v-Ha-ras* are all increased, indicate that there are early molecular changes relevant to stem cell dynamics and skin cancer. It is common for CSCs to display dysregulation of self-renewal pathways (34), similar to the overexpression of *Rac1* and *CD34* in the present study. Stem cells provide a reserve of pluripotent cells able to respond to injury, including injury by chemical insult (35). Work showing that arsenic *in vitro* can "stall" human skin stem cell differentiation, potentially creating additional target cells subsequent for carcinogenic initiation or promotion (15, 16), supports this potential for arsenic *in vivo* in the present study. The concept that carcinogenic stimuli directed at tissues with more stem cells as potential targets can lead to an intensified oncogenic response is supported by human

data from women surviving the Hiroshima and Nagasaki bombings, where the most susceptible to breast cancer were those exposed during late adolescence (5) when the mammary gland seems to have the highest number of stem cells (36).

Conclusions. Tens of millions of people worldwide are exposed to unhealthy environmental levels of inorganic arsenic (11), including, for many, exposure during the fetal life stage. Emerging data in both humans and rodents indicate that arsenic has transplacental carcinogenic potential (9, 10). Work in mice clearly shows that contemporaneous exposure is not necessary for arsenic to influence the carcinogenic process in numerous internal organs, and events *in utero* are sufficient to precipitate initiation or otherwise increase sensitivity to carcinogenesis stimulated by other agents later in life (9). In keeping with these studies, the present work shows *in utero* arsenic exposure, although by itself oncogenically quiescent, clearly facilitated skin cancer, possibly via distortion in stem cell response. The general concept that fetal toxicant exposure could be dormant and yet affect the development of cancer by altering the response to a chemical insult occurring much later in life could have broad implications in cancer susceptibility. This is particularly true for a widespread environmental toxicant such as inorganic arsenic.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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