Arsenic is a human pulmonary carcinogen. Our work indicates that in utero arsenic exposure in mice can induce or initiate lung cancer in female offspring. To define early molecular changes, pregnant C3H mice were given 85 ppm arsenic in drinking water from days 8 to 18 of gestation and expression of selected genes in the fetal lung or in lung tumors developing in adults was examined. Transplacental arsenic exposure increased estrogen receptor-α (ER-α) transcript and protein levels in the female fetal lung. An overexpression of various estrogen-regulated genes also occurred, including trefoil factor-3, anterior gradient-2, and the steroid metabolism genes 17-β-hydroxysteroid dehydrogenase type 5 and aromatase. The insulin growth factor system, which can be influenced by ER and has been implicated in the pulmonary oncogenic process, was activated in fetal lung after gestational arsenic exposure. In utero arsenic exposure also induced overexpression of α-fetoprotein, epidermal growth factor receptor, L-myc, and metallothionein-1 in fetal lung, all of which are associated with lung cancer. Lung adenoma and adenocarcinoma from adult female mice exposed to arsenic in utero showed widespread, intense nuclear ER-α expression. In contrast, normal adult lung and diethylnitrosamine-induced lung adenocarcinoma showed little evidence of ER-α expression. Thus, transplacental arsenic exposure at a carcinogenic dose produced aberrant estrogen-linked pulmonary gene expression. ER-α activation was specifically associated with arsenic-induced lung adenocarcinoma and adenoma but not with nitrosamine-induced lung tumors. These data provide evidence that arsenic-induced aberrant ER signaling could disrupt early life stage genetic programing in the lung leading eventually to lung tumor formation much later in adulthood.

Key Words: inorganic arsenic; transplacental exposure; lung cancer; estrogen signaling.

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show the lung is a target of arsenic carcinogenesis (IARC, 2004; NRC, 1999). Gestation is a period of high sensitivity to chemical carcinogenesis in rodents and humans (Anderson, 2004), and the lung is a common target of transplacental carcinogens (Anderson et al., 2000). Since inorganic arsenic readily crosses the human and rodent placenta and enters the fetal system (Devesa et al., 2006; NRC, 1999), in utero exposure to arsenic likely occurs in human populations exposed to elevated environmental arsenic and is a plausible mode of human exposure (Smith et al., 2006). Accumulating evidence indicates that lung cancer can result from multiple changes in the genome of susceptible pulmonary cells caused by carcinogenic insult (Sekido et al., 2003). There are a variety of ways arsenic can alter gene expression (Kitchin, 2001; Rossman, 2003; Waalkes et al., 2004a), which can be associated with acquisition of aberrant cellular phenotypes (Achanzar et al., 2002; Benbrahim-Tallaa et al., 2005). We recently assessed aberrant gene expression in liver samples from male mice bearing hepatocellular carcinoma induced by in utero arsenic exposure (Liu et al., 2004, 2006; Waalkes et al., 2004a). Remarkably, disruption of estrogen signaling was observed as a key event in transplacental arsenic-induced hepatocarcinogenesis (Liu et al., 2006; Waalkes et al., 2004a). Interestingly, arsenic acted as a complete transplacental lung carcinogen only in female mice (Waalkes et al., 2003), although it was an effective lung tumor initiator in both males and females (Waalkes et al., 2004b).

In recent years, lung cancer has become increasingly common in women and evidence has potentially implicated estrogen in lung carcinogenesis (Gasperino and Rom, 2004; Stabile and Siegfried, 2004). For instance, data that indicate that the female gender carries a significantly elevated risk for lung cancer in cigarette smokers are emerging (Gasperino and Rom, 2004). It is suspected that the combination of carcinogen exposure, genetics, and endocrine-related factors, including potentially estrogen receptor-α (ER-α) (Stabile and Siegfried, 2004; Stabile et al., 2005), contribute to the female disparity in exposure-adjusted lung cancer risk (Gasperino and Rom, 2004). Thus, the present work examined early molecular changes, focusing on estrogen signaling, in fetal lung and subsequent lung tumors developing after in utero arsenic exposure in female mice.

MATERIALS AND METHODS

Animal treatment and fetal tissue collection. The current study was performed, in part, using lung samples from gestation day 18 female fetal C3H mice collected after transplacental arsenic exposure to a dose (85 ppm) shown to either produce lung adenocarcinoma formation in adulthood (Waalkes et al., 2003) or initiate lung tumor formation promotable by TPA (Waalkes et al., 2004b). Animal care was provided in accordance with the U.S. Public Health Policy on the Care and Use of Animals, and the study proposal was approved by Institutional Animal Care and Use Committee of National Cancer Institute (NCI). Pregnant C3H mice (n = 6) were obtained from the Animal Production Area, NCI–Frederick, Animal Program (Frederick, MD). A basal diet (NIH-31 Open Formula, 6% Modified; Teklad Standard Diets, Madison, WI) and water containing 85 ppm arsenic as sodium arsenite (Sigma Chemical Co, St Louis, MO) or unaltered water (control) were provided ad libitum from days 8 to 18 of gestation. The dose of arsenic used did not affect water consumption or the body weight of dams. On gestation day 18, dams and fetuses were killed and the lungs were collected from female fetuses. Samples were frozen in liquid nitrogen and stored at − 80°C until analysis. Samples of fully formed lung adenoma or adenocarcinoma induced by in utero arsenic exposure in adult female C3H were obtained from tissues taken in our prior study (Waalkes et al., 2003) and were used for immunohistochemistry (see below).

Gene expression analysis. Total RNA was isolated from frozen fetal lung samples using TRIzol reagent (Invitrogen, Carlsbad, CA) followed by purification on RNeasy columns (Qiagen, Palo Alto, CA). RNA was then reverse transcribed with MuLV reverse transcriptase and Oligo-dT primers. The forward and reverse primers for selected genes were designed using Primer Express software (Applied Biosystems, Foster City, CA). Primers for select genes were listed in Table 1 and synthesized by Sigma-Genosys (Woodlands, TX). The SYBR green DNA PCR kit (Applied Biosystems) was used for real-time polymerase chain reaction analysis. The relative differences in expression between groups were expressed using cycle time (Ct) values as follows: the Ct values of the interested genes were first normalized with β-actin of the same sample, and then the relative transcript levels are expressed as percent control with control being set at 100%.

Western blot analysis. Fetal lung samples were homogenized (1:20, wt/vol) in PER-Tissue protein extraction buffer (Pierce, Rockford, IL) and freshly added protease inhibitors cocktail (CalBiochem, La Jolla, CA) and 500μM phenylmethylsulfonyl fluoride. Cytosols were prepared by centrifugation at 12,000 × g for 10 min at 4°C. Protein concentrations were determined using the dye-binding assay (Bio-Rad, Hercules, CA). Total protein (30 μg) was subjected to electrophoresis on Nupage gels (4–12%) (Invitrogen), followed by electrotheretic transfer to nitrocellulose membranes at 30 V for 1 h. Membranes were blocked in 5% dried milk in TBST (15mM Tris-HCl, pH 7.4, 150mM NaCl, and 0.05% Tween 20) for 2 h at room temperature, followed by incubation with the first antibody (1:200 to 1:1000) in Blotto (Pierce) overnight at 4°C. After three washes with TBST, the membranes were incubated in secondary antibody (1:4000 to 1:10,000) for 45 min. After four washes with TBST, proteins were visualized using SuperSignal substrate (Pierce).

Immunohistochemical localization for ER-α. Representative lung adenocarcinomas or adenomas induced by transplacental arsenic exposure in female mice (Waalkes et al., 2003) were compared with normal lung or with lung carcinomas induced by diethylnitrosamine (DEN) from female mice. A polyclonal antibody against ER-α (Santa Cruz Biotechnology, Santa Cruz, CA) was used as the primary antibody (diluted 1:1000) together with a streptavidin-conjugated secondary antibody (Santa Cruz Biotechnology). Antibody binding was visualized with an avidin-biotin-peroxidase kit (VECTASTAIN Elite ABC Kit; Vector Laboratories, Burlingame, CA) with diaminobenzidine as the chromagen and hematoxylin as a nuclear counterstain.

Statistics. Means and SEMs were calculated, and comparisons between groups were made by the Student’s t-test. Fischer’s exact test was used to analyze the incidence of lungs immunohistochemically positive for ER-α expression. A two-sided probability level of p < 0.05 was considered significant.

RESULTS

Estrogen Receptor and Related Gene Expression in Female Fetal Lung After In Utero Arsenic Exposure

Prior work showed that transplacental arsenic exposure in C3H mice induces a marked increase in lung adenocarcinoma incidence in female offspring as adults (Waalkes et al., 2003).
Thus, the exposure protocol producing lung cancers (85 ppm arsenic in drinking water from days 8 to 18 of gestation) was duplicated, and female fetal lungs were collected for analysis. Genes were selected based on prior work showing aberrant estrogen signaling associated with transplacental arsenic hepatocarcinogenesis (Waalkes et al., 2004a) and based on relevance to pulmonary carcinogenesis. Figure 1 shows the expression of ER-α and ER-β, as well as the ER-linked genes trefoil factor-3 (Tff3) and anterior gradient-2 (Agr2). In addition, genes encoding for enzymes important in steroid metabolism such as 17-β-hydroxysteroid dehydrogenase type 5 (17β-HSD5) and aromatase were assessed. Compared with control, a dramatic 530% increase occurred in pulmonary ER-α transcript in female fetuses after gestational exposure to arsenic. The expression of ER-β in arsenic-treated fetal lung was unchanged. The expression of various estrogen-related genes was markedly increased, including Tff3 (966%), Agr2 (321%), 17β-HSD5 (355%), and aromatase (253%). In marked contrast, the expression of ER-α or these ER-linked genes was unchanged in male fetal lung as compared with control (data not shown).

To help confirm the results of transcript analysis, western blot analysis was performed in control and arsenic-treated fetal lung on selected proteins (Fig. 2). ER-α protein was increased in fetal lung by arsenic exposure, which is in accord with increases seen in the transcript. The potential oncogene and tumor cell marker, α-fetoprotein (AFP), was also markedly increased at the protein level in arsenic-treated fetal lung.

**Table 1**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession number</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFP</td>
<td>V00743</td>
<td>AGCTCAAGGGAGAGAATGTGTT</td>
<td>GTTGACAGGGGCTTCTCTT</td>
</tr>
<tr>
<td>Agr2</td>
<td>NM_011783</td>
<td>CTTCTGGCTCACAAGAAGAATG</td>
<td>ATGGCCCAAGGAGCAGGAGAT</td>
</tr>
<tr>
<td>Aromatase</td>
<td>D00659</td>
<td>CCGTGGTCTGGTCGAAGAAGAATG</td>
<td>TGAGGTCACACACATCCACAG</td>
</tr>
<tr>
<td>β-Actin</td>
<td>M12481</td>
<td>GGGCAACCCGTGAAAAAGATGA</td>
<td>CAGGCTGGATGGCTACGTACA</td>
</tr>
<tr>
<td>EGFR</td>
<td>X78987</td>
<td>CCTTGGAGGTGGCTCTATTC</td>
<td>TCCAGAGCTCTCTCTCTTGA</td>
</tr>
<tr>
<td>ER-α</td>
<td>M38651</td>
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<td>TGCAGAGCTCTCTCTCTT</td>
</tr>
<tr>
<td>ER-β</td>
<td>NM_207077</td>
<td>CAGGTTGATGGCCGAAACAA</td>
<td>CAGGCTGGTCTGGGACTT</td>
</tr>
<tr>
<td>17β-HSD5</td>
<td>NM_030611</td>
<td>GCGTGAGGAATGTCAGAAGA</td>
<td>GTGGCAACAGGGCTTCTT</td>
</tr>
<tr>
<td>IGF-1</td>
<td>X04480</td>
<td>TCTTGGAGGAGGTGGGAATT</td>
<td>AGGCGGGCTGTTTCTT</td>
</tr>
<tr>
<td>IGF-2</td>
<td>M14951</td>
<td>AGAGGTGACAGAGGGCAACAG</td>
<td>TTGCTGGACATCTCCGAG</td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>X81579</td>
<td>TGGAGAGGATGGCGATG</td>
<td>TGGATGGGTTCACACAG</td>
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<tr>
<td>IGFBP-5</td>
<td>X81583</td>
<td>CCCAAGCACACTGGCATTT</td>
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<tr>
<td>IGFR2</td>
<td>U04710</td>
<td>TTTGGAGGATGGCATAG</td>
<td>AGGGGAAGATCACCATC</td>
</tr>
<tr>
<td>L-myc, lung-specific myc oncogene</td>
<td>X13945</td>
<td>CCCATACCTCCCCCTTTC</td>
<td>GGCGCAATCACCCATA</td>
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<tr>
<td>MT-1</td>
<td>BC027622</td>
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<td>GCTGGGTTGTCGAGAATA</td>
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<tr>
<td>TFF3</td>
<td>NM_011575</td>
<td>CTCTGGAGGAGGAGCAATG</td>
<td>TGAACAGCAGGGCAT</td>
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**FIG. 1.** Expressions of ER-α, ER-β, Tff3, Agr2, 17β-HSD5 and aromatase transcripts in gestation day 18 female fetal mouse lung after in utero arsenic exposure. Data are expressed as mean ± SEM (n = 6). *Significantly different (p ≤ 0.05) from control.

**FIG. 2.** Western blot analysis of selected proteins in control fetal lung (lanes 1 and 2) and arsenic-exposed fetal lung (lanes 3 and 4). The kilodalton sizes are ER-α (≈ 70 kDa), AFP (≈ 75 kDa), and β-actin (≈ 43 kDa).
including those for the epidermal growth factor receptor (EGFR, 3.2-fold), AFP (6.9-fold), L-myc (1.9-fold), and metallothionein-1 (MT-1, 2.1-fold) genes. EGFR, AFP, and L-myc are all potentially important in lung cancer (Cappuzzo et al., 2005; Hiroshima et al., 2002; Shih et al., 2002). In fact, overexpression of EGFR and AFP can be associated with particularly aggressive lung malignancies (Cappuzzo et al., 2005; Hiroshima et al., 2002). These very early expression changes in lung oncogenesis–related genes may be relevant to the arsenic-induced carcinogenic process that results in lung cancer formation much later in adulthood.

Insulin Growth Factor–Related Gene Expression in Female Fetal Lung Exposed to Arsenic In Utero

Transcript levels of genes encoding for the insulin growth factor (IGF) system, which can be influenced by ER and have also been implicated in the lung oncogenic process (Pavelic et al., 2005), are shown in Figure 4. The expression of both IGF-1 and -2 was increased approximately 2-fold by in utero arsenic exposure. In addition, IGF receptor-1 (IGFR1; 2.4-fold) and IGFR2 (1.8-fold) were similarly increased. Expression of IGF-binding protein 1 (IGFBP-1; 2.5-fold) and IGFBP-5 (1.6-fold) was also increased by in utero arsenic exposure.

To determine if ER-α overexpression was related to arsenic-induced lung cancer in female offspring, lung adenocarcinomas resulting from gestational arsenic exposure were studied immunohistochemically. A typical arsenic-induced lung adenocarcinoma stained with hematoxylin and eosin (H&E) is shown for reference (Fig. 5A). When stained for ER-α, the lung adenocarcinomas induced by in utero arsenic exposure showed intense and widespread expression of ER-α that was particularly pronounced in tumor cell nuclei (Fig. 5B). A nuclear localization is thought to represent an active form of this receptor. ER-α overexpression appeared to be common at all stages of lung tumor development induced by in utero arsenic exposure in female mice as evidenced by the intense and widespread staining for ER-α in a typical arsenic-induced lung adenoma (Fig. 6A). To see if ER-α expression was specific to arsenic, a lung adenocarcinoma induced by DEN was also examined (Fig. 6B). There was little immunohistochemical evidence of ER-α expression in the DEN-induced pulmonary adenocarcinoma.

The rate of positive ER-α expression in mouse lung tumors induced by in utero arsenic exposure or DEN treatment in adulthood as assessed immunohistochemically is shown in Table 2. Most of arsenic-induced lung tumors were strongly positive for ER-α (83%), while all DEN-induced lung tumors examined were negative.

DISCUSSION

The present study clearly showed that in utero arsenic exposure at a level that affects pulmonary tumor development in adulthood increased expression of ER-α and genes related to estrogen signaling in the fetal lung of female mice. In lung adenoma or adenocarcinoma occurring in adult mice exposed to arsenic in utero, ER-α protein overexpression was widespread and intense, indicating that in utero arsenic induced aberrant ER signaling in the lung in a fashion similar to that seen during transplacental arsenic hepatocarcinogenesis (Waalkes et al., 2004a). Thus, this study further fortifies the hypothesis that aberrant estrogen signaling is associated with transplacental arsenic carcinogenesis, at least in some tissues (Liu et al., 2004, 2006; Waalkes et al., 2004a, 2006a,b). Our recent results in CD1 mice indicate that urinary bladder proliferative lesions, including tumors, associated with prenatal arsenic exposure are greatly enhanced by postnatal exposure to estrogenic agents (Waalkes et al., 2006a,b). As a corollary in humans, increased mortality occurs from lung cancers in young adults following in utero exposure to elevated levels of arsenic in the drinking water (Smith et al., 2006). Thus, the developing fetus appears to be quite sensitive to arsenic-induced carcinogenesis.

Lung cancer is the leading cause of cancer death in the United States and, once diagnosed, often has a poor prognosis.
Accumulating evidence indicates that females may be more sensitive to insult by lung carcinogens (Gasperino and Rom, 2004). In human, lung cancer is associated with inorganic arsenic exposure either from drinking water or by inhalation (IARC, 2004; NRC, 1999). A recent study showed that chronic oral exposure to inorganic arsenate increased lung tumor formation in A/J mice (Cui et al., 2006). Inorganic arsenic can also act as a complete transplacental lung carcinogen or as a lung tumor initiator in female mice (Waalkes et al., 2003, 2004b). A variety of clinical and experimental data suggest that aberrant estrogen signaling plays a role in lung cancer (Gasperino and Rom, 2004; Patel, 2005; Stabile and Siegfried, 2004; Stabile et al., 2005). A study in humans found that ER-α expression occurs more often in the lungs and in lung tumors of women than men, and this potential gender-dependent difference could contribute unique phenotypic characteristics to lung cancer development and progression in women (Fasco et al., 2002). The present study shows that in utero exposure to the carcinogenic dose of inorganic arsenic (Waalkes et al., 2003, 2004b) resulted in the overexpression of pulmonary ER-α in female fetus. ER-α overexpression was clearly associated with arsenic-induced lung adenoma and adenocarcinoma in adult females treated with arsenic during prenatal life. In fact, arsenic-induced lung tumors that arise in adults after gestation exposure show widespread overexpression of ER-α which is particularly intense in the tumor cell nuclei, the site where this transcription factor is normally active (Hart and Davie, 2002). In contrast, ER-α expression was very poor in normal lung tissue or in DEN-induced lung adenocarcinoma, indicating that ER-α expression may be specific to arsenic, rather than typical for mouse pulmonary tumors in general.

The overexpression of pulmonary ER-α observed in the present work is consistent with our earlier observation of uterine (Waalkes et al., 2006b) or hepatic (Waalkes et al., 2004a) proliferative lesions, including tumors, induced by in utero arsenic exposure. In addition, in utero arsenic exposure combined with postnatal exposure to diethylstilbestrol greatly increases urogenital cancers in female CD1 mice and enhances expression of estrogen-regulated genes in neonatal uterus (Waalkes et al., 2006b). Furthermore, prenatal arsenic plus postnatal diethylstilbestrol or tamoxifen increases urinary bladder transitional cell hyperplasia and tumors that show clear

**FIG. 5.** Immunohistochemical analysis of mouse ER-α expression in lung adenocarcinoma in adult female mice induced by in utero arsenic exposure. (A) Morphology of arsenic-induced lung adenocarcinoma (hematoxylin and eosin, ×400). (B) Immunohistochemical analysis for ER-α expression in a typical arsenic-induced lung adenocarcinoma (×400). The lung adenocarcinoma shows widespread and intense expression for ER-α (brown staining), particularly in the tumor cell nuclei.

**FIG. 6.** Immunohistochemical analysis of ER-α expression of mouse lung tumors induced by in utero arsenic exposure or DEN treatment in adulthood. (A) ER-α expression in a typical lung adenoma (×100) induced by in utero arsenic exposure showing widespread and intense expression for ER-α (brown staining), particularly in the tumor cell nuclei. (B) ER-α expression in a DEN-induced lung adenocarcinoma (×100) showing little or no expression.
The overexpression of oncogenesis-related genes in arsenic-exposed fetal lung is noteworthy and includes overexpression of AFP, L-myc, and EGFR, which have been all implicated in lung carcinogenesis. For instance, elevated serum levels of AFP occur in patients with lung carcinoma, and primary lung carcinoma can overexpress AFP (Hiroshima et al., 2002). AFP is known as a growth-promoting oncoprotein often overexpressed in embryonic tumors (Mizejewski and MacColl, 2003) that can regulate tumor growth (Mizejewski, 2002). Similarly, L-myc can act as an oncogene, and overexpression and amplification of Myc family members are frequently seen in lung cancer cells or tumors (Shih et al., 2002). Data also indicate that high levels of EGFR can occur in human lung cancer (Cappuzzo et al., 2005). There is a functional link between ER and EGFR, and EGFR can be activated by estrogens and act in estrogen signaling (Stabile et al., 2005). Both AFP and EGFR overexpression is associated with particularly aggressive forms of pulmonary cancer (Cappuzzo et al., 2005; Hiroshima et al., 2002). MT is a low–molecular-weight protein implicated in adaptive responses to arsenic toxicity and transplacental carcinogenesis (Liu et al., 2006). Thus, early life exposure to arsenic is clearly activating several genes associated with lung oncogenesis.

Fetal pulmonary overexpression of various members of the IGF system was evident following prenatal arsenic exposure in the present work. The IGF system plays a critical role in the development and progression of cancer (LeRoith and Roberts, 2003). IGF is implicated in carcinogenesis through regulation of malignant cell proliferation, differentiation, and apoptosis (Ibrahim and Yee, 2004). IGF-1 in particular is linked to malignant transformation and has been recognized in both experimental and clinical settings, suggesting that the enhancement of growth factor pathways potentially could increase the risk for cancer development (Ibrahim and Yee, 2004). A growing body of epidemiological data suggests that high levels of circulating IGF-1 constitute a risk factor for the development of lung cancers (LeRoith and Roberts, 2003). Clinical data indicate that serum levels of IGF-1 and IGF-2 are elevated in lung cancer patients (Izycki et al., 2004). Disruption of the IGF/IGFR axis is also potentially involved in lung cancer formation (Pavelic et al., 2005). A recent study showed that estrogen-induced stimulation of proliferation of cancer cells is partly achieved via IGF signaling (Gielen et al., 2005). IGFBPs are regulated by estrogens and potentially play a role in the modulation of cancer cell proliferation (Gielen et al., 2005). Thus, the fetal overexpression of the IGF/IGFRs axis seen in the present work after in utero exposure to arsenic could have important implications in eventual lung tumor formation.

In summary, this study indicates that in utero exposure to inorganic arsenic at a dose that induces lung cancer in female offspring in adulthood causes a remarkable increase in expression of fetal pulmonary ER-α and ER-linked genes. In both early (adenoma) and advanced (adenocarcinoma) lung tumors developing in adult female mice exposed to arsenic in utero,
ER-α was also overexpressed. This work provides evidence that arsenic exposure may induce early life stage reprogramming of key genes in growth signaling, leading to tumor formation much later in life.

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