Published in final edited form as:

Chem Biol Interact. 2009 December 10; 182(2-3): 253–258. doi:10.1016/j.cbi.2009.07.023.

Fetal arsenic exposure appears to facilitate endocrine disruption by postnatal diethylstilbestrol in neonatal mouse adrenal

Jie Liu^a, Limei Yu^{a,b}, Jean-Francois Coppin^a, Erik J. Tokar^a, Bhalchandra A. Diwan^c, Michael P. Waalkes^{a,*}

^aInorganic Carcinogenesis Section, Laboratory of Comparative Carcinogenesis, National Cancer Institute at NIEHS, Research Triangle Park, NC, United States

^bZunyi Medical College, Zunyi, China

^cBasic Research Program, SAIC-Frederick Inc., NCI Frederick, Frederick, MD, United States

Abstract

Fetal exposure of mice to arsenic and subsequent postnatal diethylstilbestrol (DES) facilitates production of urogenital system and liver tumors in the offspring when they reach adulthood. The adrenal is a target of endocrine disruption that could influence tumor formation at other sites. Thus, we examined possible fetal arsenic-induced adrenal effects as a potential basis of arsenic enhancement of DES carcinogenesis. Pregnant CD1 mice were given drinking water containing 85 ppm arsenic as sodium arsenite or unaltered water from day 8 to day 18 of gestation and were allowed to deliver normally. Groups of offspring were subsequently injected s.c. on postpartum days 1–5 with DES (2 µg/pup/day) and killed on postnatal day 12. Total RNA was isolated from the whole adrenal glands, and the expression of various genes was analyzed by real-time RT-PCR. Fetal arsenic exposure greatly enhanced DES-induced, estrogen linked gene expression, such as estrogen receptor- α and trefoil factors. Expression of genes involved with steroid metabolism and/or methionine metabolism was also increased, including genes encoding for 17 β -hydroxysteroid dehydrogenase type 5 (*HSD17 β 5*) and androstenedione 15 α -hydroxylase (*Cyp2a4*). The transcripts for homocysteine cycling genes (betaine-homocysteine methyltransferase and thioether S-methyltransferase) and developmental marker genes (α -fetoprotein, insulin-like growth factor 2 and IGF binding protein-1), were also higher with arsenic plus DES than either treatment alone. Thus, exposure of the mouse to arsenic during a critical period of fetal development may potentially alter adrenal genetic programming, leading to endocrine disruption and potentially enhancing tumor formation together with DES at other sites much later in life. Functional studies, such as changes in circulating steroids, would greatly support this hypothesis, and are planned.

Keywords

Arsenic; Diethylstilbestrol; Carcinogenesis; Gene expression; Neonatal tissues; Adrenal

*Corresponding author at: Inorganic Carcinogenesis Section, NCI at NIEHS, Mail Drop F0-09, Research Triangle Park, NC 27709, United States. Tel.: +1 919 541 2328; fax: +1 919 541 3970. waalkes@niehs.nih.gov (M.P. Waalkes).

Conflict of interest statement
None declared.

1. Introduction

Inorganic arsenic is a human and rodent carcinogen, causing tumors of the skin, urinary bladder, lung, liver, prostate, kidney, and possibly other sites [1,2]. We have shown that short-term exposure of pregnant mice to inorganic arsenic produces a variety of internal tumors in the offspring when they reach adulthood [3–7]. Inorganic arsenic readily crosses the rodent and human placenta and enters the fetus [1,2,8,9]. Gestation is a period of high cell proliferation and a time of sensitivity to chemical carcinogenesis by some chemicals in rodents and possibly in humans [10], potentially including to inorganic arsenic [12]. Gestational arsenic exposure can act as a complete carcinogen and as a tumor initiator, as the carcinogenic effects of fetal arsenic exposure can be promoted after birth by carcinogenic estrogen, diethylstilbestrol (DES) [5,6], or the generalized tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [4,7].

The spectrum of tumors and/or proliferative lesions induced by *in utero* arsenic exposure in mice, including tumors of the liver, ovary, adrenal, uterus and urinary bladder, resembles the potential targets of carcinogenic estrogens [3–7]. This has led to the hypothesis that arsenic may, at least partially, produce some of its carcinogenic effects through aberrant estrogen signaling [11,12], and is consistent with what many would consider “endocrine disruption” [13]. The hypothesis that aberrant estrogen signaling is involved in transplacental arsenic carcinogenesis is supported by data showing overexpression of genes related to aberrant estrogen signaling in arsenic-induced tumors in adult mice [14–16], in fetal arsenic exposed lung [17], fetal arsenic treated liver [18], or in cultured fetal liver cells exposed to arsenic [19]. The enhanced carcinogenic effects seen with fetal arsenic and subsequent exposure to the synthetic estrogen DES in various sites [5,6] lends further support to an estrogen mediated mechanism. Estrogen receptor- α (ER- α) and ER-linked protein pS2 (also called Trefoil factor-1, Tff1) is clearly overexpressed in uterine adenocarcinoma and in urinary bladder transitional cell carcinoma in adult mice induced by a combined treatment with arsenic *in utero* plus postnatal DES exposure [5]. Indeed, in these mice [5], early molecular evidence of estrogen-responsive gene expression includes greatly enhanced expression of cytochrome P450 2A4 (Cyp2a4), *Tff1*, and Lactoferrin in the neonatal uterus in mice given arsenic and postnatal DES [5].

The adrenal gland is a target of endocrine disruption [13] and transplacental arsenic consistently produces adrenal cortical tumors [5,6,11–13]. It is possible that effects in the adrenal could modify tumor formation in other organs. Therefore, it was of interest to examine interactions of *in utero* arsenic exposure plus postnatal DES treatment in neonatal adrenal. Using real-time RT-PCR technique, the present study examined gene expression related to estrogen signaling and steroid metabolism. The results clearly revealed that transplacental arsenic exposure enhanced the expression of DES-inducible genes in neonatal adrenals, adding the supporting evidence for involvement of aberrant estrogen signaling in transplacental arsenic carcinogenesis.

2. Materials and methods

2.1. Chemicals

Sodium arsenite (NaAsO_2 ; 97.0% purity) and DES (99% minimum purity) were obtained from Sigma Chemical Co. (St. Louis, MO).

2.2. Animals and treatments

Timed pregnant CD1 mice were given drinking water containing 85 ppm arsenic or unaltered water ad libitum from day 8 to day 18 of gestation. This arsenic treatment protocol produced carcinogenic effects without affecting maternal and fetal growth [5,6]. After birth, on postpartum days 1–5 newborns were given s.c. injections of DES ($2\mu\text{g}/\text{pup}/\text{day}$) or vehicle (corn oil). One week after the last treatment of DES, mice were killed by CO_2 asphyxiation and whole adrenals were harvested, and frozen at -80°C until analysis. Animal care was provided in accordance with the US Public Health Policy on the Care and Use of Animals, and the Institutional Animal Care and Use Committee approved this study proposal.

2.3. Real-time RT-PCR analysis

Total RNA was isolated from frozen neonatal tissue samples using TRIzol reagent (Invitrogen, Carlsbad, CA) followed by purification on RNeasy columns (Qiagen, Polo Alto, CA). The levels of expression of the selected genes were quantified using real-time RT-PCR analysis. The forward and reverse primers for selected genes were designed using ABI Primer Express software (Applied Biosystems, Foster City, CA) and listed in Table 1. Total RNA was reverse transcribed with MuLV reverse transcriptase and oligo-dT primers, and subjected to real-time PCR analysis using Power SYBR green PCR master mix (Applied Biosystems, Cheshire, UK). The cycle time (Ct) values of genes of interest were first normalized with β -actin and 18S from the same sample, and then the relative differences between control and treatment groups were calculated and expressed as percentage of controls.

2.4. Statistics

Data are expressed as means $\pm$ SEM of five individual samples. Differences between groups were analyzed by one-way ANOVA followed by a Dunnett's *t*-test. A two-sided probability level of $p < 0.05$ was considered significant.

3. Results

3.1. Real-time RT-PCR analysis of aberrantly expressed genes in adrenals

Combined transplacental arsenic exposure plus postnatal treatment with DES in mice enhances tumors in several endocrine sensitive/responsive sites beyond additive for either treatment alone [5,6]. Since the adrenals can also be a site of endocrine disruption we sought to explore the early molecular events of prenatal arsenic exposure and postnatal DES on adrenal gland on postnatal day 12 (PND12). Total RNA was isolated, purified and subjected to gene expression analysis using real-time RT-PCR.

Fig. 1 illustrates the expression of *ER-α* and ER-linked genes Trefoil factor-3 (*Tff3*) and Anterior gradient 2 (*Agr2*). In male adrenals, the expression of *ER-α*, *Tff3* and *Agr2* was greatly enhanced in the arsenic plus DES group. In female adrenals, the expression of *Agr2* was elevated over control higher either arsenic alone and arsenic plus DES, but a synergistic effect appeared to occur in the arsenic plus DES group for *ER-α* and *Tff3* expression compared to either treatment alone.

Fig. 2 shows the expression of genes related to steroid metabolism. The basal expression of *Cyp2a4* was higher in female adrenal glands, and was increased by DES alone. When prenatal arsenic was combined with postnatal DES, the expression of *Cyp2a4* was markedly increased in both sexes. The expression of 17β-hydroxysteroid dehydrogenase type 5 (*HSD17β5*) was modestly increased by DES in females, but arsenic plus DES greatly increased *HSD17β5* expression in both male and female adrenal glands.

Fig. 3 shows the expression of genes related to methionine metabolism. The expression of betaine-homocysteine methyltransferase (*Bhmt*) and thioether S-methyltransferase (*Temt*, also called indolethylamine N-methyltransferase, *Inmt*) in males was unaffected by arsenic or DES alone in males but was increased by DES in females. When in utero arsenic was combined with postnatal DES treatment, the expression of *Bhmt* was markedly enhanced in both sexes while *Temt* was markedly increased in female offspring.

Fig. 4 shows the expression of genes related to development and cell growth. The expression of alpha fetoprotein (*Afp*) normally decreases after birth. The expression of *Afp* was unaffected by arsenic or DES alone in both sexes, but when arsenic was combined with DES, *Afp* expression was markedly enhanced, suggesting a disruption of development. The IGF axis, notably insulin-like growth factor 2 (*Igf2*) and IGF-binding proteins, plays an important role in adrenal carcinogenesis [27]. Thus, the gene expression of *Igf2* and insulin-like growth factor binding protein-1 (*Igfbp1*) was assayed. The basal expressions of *Igf2* and *Igfbp1* were largely unaffected by arsenic or DES alone. However, when arsenic was combined with DES, *Igf2* and *Igfbp1* expressions greatly increased.

4. Discussion

The present study demonstrates that *in utero* arsenic exposure plus postnatal DES treatment in mice results in interactions at the gene transcript level related to disrupted estrogen signaling (such as increased *ER-α*, *Tff3*, and *Agr2* expression), steroid metabolism (such as *Cyp2a4* and *HSD17β5*) and methionine metabolism (such as *Bhmt* and *Temt*) in neonatal adrenal gland. These data provide evidence that arsenic potentially enhances the ability of DES to cause endocrine disruption in this tissue. Many of these findings are likely related to the adrenal cortex, where transplacental arsenic causes tumors in mice [5,6,11–13]. Our initial hypothesis that inorganic arsenic might act through aberrant activation of estrogen signaling pathways [11,12] has been further supported by the synergistic effects of arsenic with the synthetic estrogen DES in enhancing tumor incidence at several in mice [5,6] and in enhancing estrogen-linked gene expression in the neonatal adrenal in the present study. The hypothesis of the current study is that arsenic may predispose tissue to interact with DES to produce endocrine disruption in the adrenal gland and thereby influence tumor formation

elsewhere. Arsenic has been shown to directly bind to a synthetic peptide constructed based on the estrogen receptor binding region of human ER- α [32], and this could be a factor in aberrant estrogen signaling, including estrogen signaling by signaling by subsequent synthetic estrogens. However, a limitation of this study is that only adrenal gene transcripts were assessed, which, although clear, do not support unambiguously disrupted function. Functional studies, such as changes in circulating or adrenal cortical steroids, would greatly support this hypothesis, and are planned.

In the neonatal adrenal, the expression of *ER- α* was normally low, and arsenic alone or DES alone had no affect adrenal *ER- α* expression. However, ER- α was increased ~200% by arsenic plus DES. Further, the expression of estrogen-linked genes (such as *Tff3* and *Agr2*) was greatly enhanced when *in utero* arsenic was combined with postnatal DES treatment. Both *Tff3* and *Agr2* are estrogen-regulated genes [21], and overexpression of *ER- α* , *Tff3*, and *Agr2* has been observed in fetal C3H lungs exposed to arsenic alone [17]. Likewise *Agr2* and *Tff1* are prominently overexpressed in fetal liver following *in utero* arsenic exposure [18]. In primary cultures of mouse fetal liver cells exposed to arsenic *in vitro*, expression of *ER- α* , *Tff3*, small proline-rich protein 2A (*Sprr2a*) and *Agr2* are all increased [18]. The expression of these genes might be persistent to adulthood, as with adult mouse uterine adenocarcinoma and urinary bladder transitional cell carcinoma induced by *in utero* arsenic plus postnatal DES which both show intense staining for *Tff1* and ER- α protein [5]. Adult C3H lung tumors and liver tumors induced by *in utero* arsenic exposure also showed intense ER- α staining [6,17]. These data help link aberrant estrogen signaling to tumors stimulated by fetal arsenic exposure.

The adrenal gland plays an important role in steroid metabolism and secretion. The expression of various genes encoding for steroid metabolizing enzymes were also greatly enhanced when arsenic was combined with DES. For example, the female-dominant *Cyp2a4* (also called androstenedione 15 α -hydroxylase) and enzymes of steroid metabolism, *HSD17 β 7* and *HSD17 β 5*, were all markedly increased in the neonatal adrenal when arsenic was combined with DES. The early life-stage is a period of high susceptibility to environmental endocrine disruptors, such as DES [22]. Although no synergy occurs in the adrenal cortical tumor formation with fetal arsenic and postnatal DES [5,6], the potential endocrine disrupting effects seen in the present study with arsenic and DES on the adrenal gland steroid gene expression might play a role in tumor formation in other organs, such as the urogenital system and liver [5,6,14–16]. Further studies are warranted to define such a potential relationship.

The expression of *Bhmt* and *Temt* were also markedly increased in the adrenal gland when arsenic was combined with DES. Altered methyl group metabolism could provide a potential mechanism for inducing epigenetic changes during perinatal period [23], and aberrant expression of genes important in methyl metabolism, including *Bhmt* and *Temt*, could impact DNA methylation. The expression of genes for homocysteine recycling is proposed not only to play an important role in DNA methylation and potentially gene imprinting [20,24]. DNA methylation is important for imprinting during development, and has been shown to be altered in the adult [11] or in the newborn [25] following *in utero* arsenic exposure.

Expression of *Afp* is high in the fetal tissues and gradually decreases during development and after birth. Overexpression of *Afp* transcript induced by arsenic plus DES in neonatal adrenals suggests a disruption of some developmental process. Aberrant expression of *Afp* in the liver and lung is evident in transplacental arsenic carcinogenesis studies in adult mice [14–16] or in mouse fetus [17,18], or in cultured fetal liver cells exposed to arsenic *in vitro* [19]. The expression of the IGF axis has been implicated in adrenal carcinogenesis, particularly the overexpression of *Igf2* [26]. IGF-2 plays an important role in fetal adrenal development [27], and aberrant expression of IGF-2 cluster is evident in adenocarcinomas [28]. Thus, the enhanced expression of IGF system and *Afp* transcripts could well play a role in adrenal carcinogenesis. Increased expression of *Igf2* and *Igfbp1* was also observed in the fetal C3H mouse lung exposed to arsenic [17]. Thus, transplacental arsenic acted with DES in altering expression of developmentally related gene expressions.

In summary, the present study demonstrates that *in utero* arsenic exposure plus postnatal DES treatment resulted in interactions in gene expression in the neonatal adrenals involving a complex interplay between steroid metabolism and estrogen signaling pathways, which may indicate early genetic reprogramming [29], consistent with endocrine disruption, leading to the formation of tumors at other sites later in life. As a corollary in humans, increased mortality occurs from lung cancers [30] and liver cancers [31] in young adults following *in utero* exposure to arsenic in the drinking water. The interactions of transplacental arsenic exposure with xenoestrogens or pharmacological estrogens may thus represent an added health risk in pregnant women.

Acknowledgements

The authors thank Drs. Wei Qu and Larry Keefer for their critical review of this manuscript. This research was supported, in part, by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research, and by National Institutes of Health, Basic Science Program under contract no. HHSN261200800001E. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

References

- [1]. NRC (National Research Council), Arsenic in the Drinking Water (Update), NRC, National Academy, Washington, DC, 2001, pp. 1–225.
- [2]. IARC (International Agency for Research on Cancer), 2004, IARC monographs on the evaluation of carcinogenic risks to humans. Vol. 84: Arsenic in drinking water In: Some Drinking Water Disinfectants and Contaminants, including Arsenic, IARC, Lyon, France, 2004, pp. 39–267.
- [3]. Waalkes MP, Ward JM, Liu J, Diwan BA, Transplacental carcinogenicity of inorganic arsenic in the drinking water: induction of hepatic, ovarian, pulmonary, and adrenal tumors in mice, *Toxicol. Appl. Pharmacol* 186 (2003) 7–17. [PubMed: 12583988]
- [4]. Waalkes MP, Ward JM, Diwan BA, Induction of tumors of the liver, lung, ovary and adrenal in adult mice after brief maternal gestational exposure to inorganic arsenic: promotional effects of postnatal phorbol ester exposure on hepatic and pulmonary, but not dermal cancers, *Carcinogenesis* 25 (2004) 133–141. [PubMed: 14514661]
- [5]. Waalkes MP, Liu J, Ward JM, Powell DA, Diwan BA, Urogenital system cancers in female CD1 mice induced by in utero arsenic exposure are exacerbated by postnatal diethylstilbestrol treatment, *Cancer Res* 66 (2006) 1337–1445. [PubMed: 16452187]
- [6]. Waalkes MP, Liu J, Ward JM, Diwan BA, Tumors and proliferative lesions of liver, urinary bladder, adrenal, lung, and kidney in male CD1 mice after transplacental exposure to inorganic

arsenic and postnatal treatment with diethylstilbestrol or tamoxifen, *Toxicol. Appl. Pharmacol* 215 (2006) 295–305. [PubMed: 16712894]

- [7]. Waalkes MP, Liu J, Germolec DR, Trempus CS, Cannon RE, Tokar EJ, Tennant RW, Ward JM, Diwan BA, Arsenic exposure in utero exacerbates skin cancer response in adulthood with contemporaneous distortion of tumor stem cell dynamics, *Cancer Res* 68 (2008) 8278–8285. [PubMed: 18922899]
- [8]. Concha G, Vogler G, Lezcano D, Nermell B, Vahter M, Exposure to inorganic arsenic metabolites during early human development, *Toxicol. Sci* 44 (1998) 185–190. [PubMed: 9742656]
- [9]. Devesa V, Adair BM, Liu J, Waalkes MP, Diwan BA, Styblo M, Thomas DJ, Speciation of arsenic in the maternal and fetal mouse tissues following gestational exposure to arsenite, *Toxicology* 224 (2006) 147–155. [PubMed: 16753250]
- [10]. Anderson LM, Diwan BA, Fear NT, Roman E, Critical windows of exposure for children's health: cancer in human epidemiological studies and neoplasms in experimental animal models, *Environ. Health Perspect* 108 (Suppl. 3) (2000) 573–594. [PubMed: 10852857]
- [11]. Waalkes MP, Liu J, Chen H, Xie Y, Achanzar WE, Zhou YS, Cheng ML, Diwan BA, Estrogen signaling in livers of male mice with hepatocellular carcinoma induced by exposure to arsenic in utero, *J. Natl. Cancer Inst* 96 (2004) 466–474. [PubMed: 15026472]
- [12]. Waalkes MP, Liu J, Diwan BA, Transplacental arsenic carcinogenesis in mice, *Toxicol. Appl. Pharmacol* 222 (2007) 271–280. [PubMed: 17306315]
- [13]. Hinson JP, Raven PW, Effects of endocrine-disrupting chemicals on adrenal function, *Best Pract. Res. Clin. Endocrinol. Metab* 20 (2006) 111–112. [PubMed: 16522523]
- [14]. Liu J, Xie Y, Ward JM, Diwan BA, Waalkes MP, Toxicogenomic analysis of aberrant gene expression in liver tumors and nontumorous livers of adult mice exposed in utero to inorganic arsenic, *Toxicol. Sci* 77 (2004) 249–257. [PubMed: 14691202]
- [15]. Liu J, Xie Y, Ducharme DMK, Shen J, Diwan BA, Merrick BA, Grissom BASF, Tucker CJ, Paules PS, Tennant RMP, Waalkes MP, Global gene expression associated with hepatocarcinogenesis in adult male mice induced by *in utero* arsenic exposure, *Environ. Health Perspect* 114 (2006) 404–411. [PubMed: 16507464]
- [16]. Liu J, Xie Y, Merrick BA, Shen J, Ducharme DMK, Collins J, Diwan BA, Logsdon D, Waalkes MP, Transplacental arsenic plus postnatal 12-*O*-teradecanoyl phorbol-13-acetate exposures induced similar aberrant gene expressions in male and female mouse liver tumors, *Toxicol. Appl. Pharmacol* 213 (2006) 216–223. [PubMed: 16368122]
- [17]. Shen J, Liu J, Xie Y, Diwan BA, Waalkes MP, Fetal onset of aberrant gene expression relevant to pulmonary carcinogenesis in lung adenocarcinoma development induced by *in utero* arsenic exposure, *Toxicol. Sci* 95 (2007) 313–320. [PubMed: 17077188]
- [18]. Liu J, Xie Y, Cooper R, Ducharme DMK, Tennant R, Diwan BA, Waalkes MP, Transplacental exposure to inorganic arsenic at a hepatocarcinogenic dose induces expression changes in mice indicative of aberrant estrogen signaling and disrupted steroid metabolism, *Toxicol. Appl. Pharmacol* 220 (2007) 284–291. [PubMed: 17350061]
- [19]. Liu J, Yu L, Tokar EJ, Bortner C, Sifre MI, Sun Y, Waalkes MP, Arsenic-induced aberrant gene expression in fetal mouse primary liver-cell cultures, *Ann. N. Y. Acad. Sci* 1140 (2008) 368–375. [PubMed: 18991936]
- [20]. Benkhalifa M, Montjean D, Cohen-Bacrie P, Ménéz Y, Imprinting: RNA expression for homocysteine recycling in the human oocyte. *Fertil. Steril*, in press.
- [21]. Wilson CL, Sims AH, Howell A, Miller CJ, Clarke RB, Effects of oestrogen on gene expression in epithelium and stroma of normal human breast tissue, *Endocr. Relat. Cancer* 13 (2006) 617–628. [PubMed: 16728587]
- [22]. Birnbaum LS, Fenton SE, Cancer and developmental exposure to endocrine disruptors, *Environ. Health Perspect* 111 (2003) 389–394. [PubMed: 12676588]
- [23]. Steele W, Allegrucci C, Singh R, Lucas E, Priddle H, Denning C, Sinclair K, Young L, Human embryonic stem cell methyl cycle enzyme expression: modeling epigenetic programming in assisted reproduction? *Reprod. Biomed* 10 (2005) 755–766.
- [24]. Pajares MA, Pérez-Sala D, Betaine homocysteine S-methyltransferase: just a regulator of homocysteine metabolism? *Cell Mol. Life Sci* 63 (23) (2006) 2792–2803. [PubMed: 17086380]

- [25]. Xie Y, Liu J, Benbrahim-Tallaa L, Ward JM, Logsdon DD, Diwan BA, Waalkes MP, Aberrant DNA methylation and gene expression in livers of newborn mice transplacentally exposed to a hepatocarcinogenic dose of inorganic arsenic, *Toxicology* 236 (2007) 7–15. [PubMed: 17451858]
- [26]. Fottner Ch., Hoeflich A, Wolf E, Weber MM, Role of the insulin-like growth factor system in adrenocortical growth control and carcinogenesis, *Horm. Metab. Res* 36 (2004) 397–405. [PubMed: 15241731]
- [27]. Coulter CL, Fetal adrenal development: insight gained from adrenal tumors, *Trends Endocrinol. Metab* 16 (2005) 235–242. [PubMed: 15949953]
- [28]. de Fraipont F, El Atifi M, Cherradi N, Le Moigne G, Defaye G, Houlgatte R, Bertherat J, Bertagna X, Plouin PF, Baudin E, Berger F, Gicquel C, Chabre O, Feige JJ, Gene expression profiling of human adrenocortical tumors using complementary deoxyribonucleic acid microarrays identifies several candidate genes as markers of malignancy, *J. Clin. Endocrinol. Metab* 90 (2005) 1819–1829. [PubMed: 15613424]
- [29]. Cook JD, Davis BJ, Cai SL, Barrett JC, Conti CJ, Walker CL, Interaction between genetic susceptibility and early life environmental exposure determines tumor suppressor gene penetrance, *Proc. Natl. Acad. Sci. U.S.A* 102 (2005) 8644–8649. [PubMed: 15937110]
- [30]. Smith AH, Marshall G, Yuan Y, Ferreccio C, Liaw J, von Ehrenstein O, Steinmaus C, Bates MN, Selvin S, Increased mortality from lung cancer and bronchiectasis in young adults after exposure to arsenic in utero and in early childhood, *Environ. Health Perspect* 114 (2006) 1293–1296. [PubMed: 16882542]
- [31]. Liaw J, Marshall G, Yuan Y, Ferreccio C, Steinmaus C, Smith AH, Increased childhood liver cancer mortality and arsenic in drinking water in northern Chile, *Cancer Epidemiol. Biomarkers Prev* 17 (2008) 1982–1987. [PubMed: 18708388]
- [32]. Kitchin KT, Wallace K, Arsenic binding to synthetic peptides based on the Zn finger region and the estrogen binding region of human estrogen receptor-alpha, *Toxicol. Appl. Pharmacol* 206 (2005) 66–72. [PubMed: 15963345]

Fig. 1.

Expression of estrogen-related genes in adrenal glands of neonatal (PND12) CD1 mice exposed to arsenic (85 ppm) *in utero* plus diethylstilbestrol treatments (DES, 2 µg/pup/day, s.c. on postpartum days 1–5). Data are mean ± SEM of five mice. *Significantly different from untreated controls; #significantly different from DES alone.

Fig. 2.

Expression of genes related to steroid metabolism in adrenal glands of neonatal (PND12) CD1 mice exposed to arsenic (85 ppm) *in utero* plus diethylstilbestrol treatments (DES, 2 µg/pup/day, s.c. on postpartum days 1–5). Data are mean ± SEM of five mice. *Significantly different from untreated controls; #significantly different from DES alone.

Fig. 3.

Expression of genes related to methionine metabolism in adrenal glands of neonatal (PND12) CD1 mice exposed to arsenic (85 ppm) *in utero* plus diethylstilbestrol treatments (DES, 2 µg/pup/day, s.c. on postpartum days 1–5). Data are mean ± SEM of five mice.

*Significantly different from untreated controls; #significantly different from DES alone.

Fig. 4.

Expression of genes related to development and growth factors in adrenal glands of neonatal (PND12) CD1 mice exposed to arsenic (85 ppm) *in utero* plus diethylstilbestrol treatments (DES, 2 µg/pup/day, s.c. on postpartum days 1–5). Data are mean ± SEM of five mice.

*Significantly different from untreated controls; #significantly different from DES alone.

Table 1

Primer sequences for real-time RT-PCR analysis.

Gene	GenBank number	Forward	Reverse
<i>Afp</i>	V00743	AGCTCAGCGAGGAGAAATGGT	GTTCACAGGGCTTGCTTCATTG
<i>Agr2</i>	NM_011783	CCTTGCGGCTCACAAAG	ATGGCCACAAGAAGCAGGAT
<i>β-actin</i>	M12481	GTATGACTCCACTCACGGCAA	GGTCTCGCTCTGTGGAAGATG
<i>Bhmt</i>	AF033381	CACATCAGGGCGATTGCA	TCCCCAGCTGCCATGTTT
<i>Cyp2a4</i>	J03549	GGAAGACGAACGGTGCTTTC	CCCGAAGACGATTGAGCTAATG
<i>ER-α</i>	M38651	TCTCTGGAAGAGAAAGGACCACATC	TGCAGAGTCAGGCCAGCTTT
<i>HSD17β5</i>	NM_030611	GCCATGGAGAAATGCAAGGA	GTTGCACACAGGCTTGCTTGAG
<i>Igf2</i>	M14951	AGAGTTCAGAGAGGCCAAACGT	TTGCTGGACATCTCCGAAGAG
<i>Igfthpl</i>	X81579	TGGACAGCTTCCACCTGATG	TGATGGCGTTCCACAGGAT
<i>Temt</i>	NM_009349	CCTACGACTGTGTCCTCCATAGTG	CTTCTGAGCTTGGCTTCTCTCT
<i>Tf23</i>	NM_011575	CTCTGTCACATCGGAGCAGTGT	TGAAAGCACCAAGGGCACATT