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A keratin 18 transgenic zebrafish Tg(k18(2.9):RFP) treated with inorganic arsenite reveals visible overproliferation of epithelial cells

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Abstract

Inorganic arsenic has strong human carcinogenic potential, but the availability of an animal model to study toxicity is extremely limited. Here, we used the transgenic zebrafish line Tg(k18(2.9):RFP) as an animal model to study arsenite toxicity. This line was chosen because the red fluorescent protein (RFP) is expressed in stratified epithelia (including skin), due to the RFP reporter driven by the promoter of the zebrafish keratin 18 gene. We titrated doses of inorganic arsenite for zebrafish embryos and found that arsenite exposure at 50 μM for 120 h was suitable for mimicking a long-term, chronic effect. When embryos derived from Tg(k18(2.9):RFP) adults were treated with this arsenite dose and time of exposure, abnormal phenotypes were not noticeable under the light microscope. However, arsenic keratosis was visible in the epithelial cells under the fluorescent microscope. Morphological defects became more severe with increased dose and exposure duration, suggesting that the severity of skin lesions was dose- and time-dependent. Histochemical examination of keratosis after 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) staining showed that the epithelial cells overproliferated after treatment with arsenite. Therefore, this Tg(k18(2.9):RFP) zebrafish line is an excellent model for studying toxicity induced by inorganic arsenite and may have potential for studying other environmental pollutants.
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1. Introduction

Arsenic toxicity has become a global health issue in the past decade. People who drink arsenic-contaminated water have an increased risk of skin, lung, and bladder cancers (Maharjan et al., 2005; Tseng et al., 2005). The primary route of arsenic exposure is from inhalation, absorption through the skin, and ingestion of contaminated water. The clinical features of arsenic toxicity vary, depending on acute or chronic exposure (reviewed by Ratnaike, 2003). In acute poisoning, the lethal dose of arsenite ranges from 100 to 300 mg. In chronic poisoning, long-term exposure leads to multisystem diseases, involving the skin and gastrointestinal, cardiovascular, neurological, genitourinary, and respiratory systems. Chronic arsenic exposure is of concern mainly because of its carcinogenic effects.

Arsenic is classified as organic and inorganic. Organic arsenic has strong human carcinogenic potential (Morales et al., 2000; Basu et al., 2001; Waalkes et al., 2004a), but inorganic arsenic is considered a

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"paradoxical" human carcinogen, because the animal models resulting in cancer show rather inconsistent findings (Basu et al., 2001; Waalkes et al., 2004b). In rats, negative results are reported when the exposure is through diet (Kanisawa and Schroeder, 1969) or drinking water (Kroes et al., 1974), whereas, stomach adenocarcinomas are detected when the inorganic arsenic is surgically implanted in the stomach (Wang et al., 2002). In mice, inorganic arsenic is termed a co-carcinogen (Popovicova et al., 2000; Rossman et al., 2001), a cancer promoter (Germolec et al., 1998), a carcinogen (Chen et al., 2000), and a transplacental carcinogen (Waalkes et al., 2004a), suggesting that carcinogenicity may differ depending on the species, the route, the dose, and the duration of exposure. Chronic exposure leads to multisystem diseases, and the most obvious consequence is skin lesions (Rossman, 2003). Dermatological changes are a common feature and are the key criteria for initial clinical diagnosis of arsenic toxicity. In the mammalian system, however, it is very difficult to observe the very subtle changes in skin after arsenic exposure. Therefore, development of an alternative animal model is essential for studying chronic arsenic poisoning.

Recently, the zebrafish has become a good model for many studies because of the large number of transparent embryos (Nusslein-Volhard and Dahm, 2002). To develop an aquatic animal model for studying inorganic arsenic-induced toxicity, we chose the zebrafish transgenic line Tg(k18(2.9):RFP), which expresses red fluorescent protein (RFP) in stratified epithelia (Wang et al., 2006). We performed a series of time- and dose-dependent arsenite exposure experiments. Subtle changes in the stratified epithelia are easily observed. This is an excellent model for studying toxicity induced by inorganic arsenite and may have potential for studying other environmental pollutants.

2. Materials and methods

2.1. Experimental fish

Zebrafish of the AB strain (wild-type, wt) were kept under a 14-h light and 10-h dark photoperiod at approximately 28.5 °C. After fertilization, the eggs were collected and cultured in an aquarium. Embryonic cleavage and somite formation were observed with a light microscope to determine developmental stage (Kimmel et al., 1995). For arsenite exposure experiments, we bred and used the transgenic line Tg(k18(2.9):RFP), which contains an upstream 2.9 kb segment of the zebrafish k18 gene fused with RFP cDNA and is able to recapitulate the endogenous k18 expression pattern (Wang et al., 2006). The maintenance procedures of this transgenic line are the same as with wt zebrafish.

2.2. Arsenite exposure

Sodium arsenite ($NaAsO_2$, Sigma) was dissolved in sterile distilled water to the desired concentrations. For dose titration, wt embryos at 72 h postfertilization (hpf) were collected, randomly divided into four groups of 80 embryos each, and exposed to either water (mock control) or water containing sodium arsenite (0.5, 5, and 50 μM). For observing skin lesions, Tg (k18(2.9):RFP) embryos at 72 hpf were collected, randomly divided into three groups of 80 embryos each, and exposed to sodium arsenite at concentrations of 0, 5, and 50 μM. All embryos were cultivated in six-well cell culture plates, and skin was examined daily until 5 days after exposure to detect morphological changes as revealed by red fluorescence.

2.3. Histology

At 24 hpf, the embryos were fixed in 4% phosphate-buffered saline (PBS)-buffered paraformaldehyde for 4 h, embedded in paraffin, and sectioned at 6 mm through the region containing the arsenic keratosis. The sections were then dewaxed and processed for hematoxylin and eosin staining by using standard techniques (Tucker et al., 2001).

2.4. Nuclei staining

Embryos were fixed in 4% PBS-buffered paraformaldehyde for 4 h at room temperature, washed once with PBS, resuspended in PBS containing 0.1% Triton × (PBTw), and incubated for 10 min on ice. To visualize nuclei, we incubated the fixed embryos with 100 ng/ml 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI, Sigma) in PBS for 30 min and rinsed them three times with PBTw. The morphology of the cells' nuclei was observed using a fluorescence microscope (Leica) at an excitation wavelength of 350 nm. Nuclei were considered to have the normal phenotype if they were glowing brightly and homogenously.

2.5. Microscopy

Transgenic embryos were observed hourly, especially from 1 to 14 hpf, under a stereo dissecting microscope (MZ12, Leica) equipped with a fluorescent module having a DsRed filter cube (Kramer Scientific). Photographs of embryos at specific stages were taken with an S2 Pro digital camera (Fuji).

2.6. Statistical analysis

Fertilization and hatching rates were analyzed statistically with the general linear models procedure (SAS, Inc., Cary, NC, USA). A one-way analysis of variance was used to compare the mean values between the mock-treated and the experimental groups. A Duncan's multiple range test was used to separate sample means. A significance level of $P < 0.01$ was used in all statistical analyses.

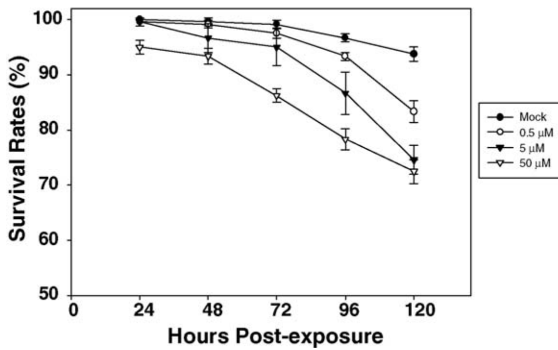

Fig. 1. Arsenite causes gradual death of zebrafish embryos in a dose-dependent manner. The *x*- and *y*-axes represent the stages after inorganic arsenite exposure and the survival rates, respectively. The experiments were performed in triplicate, and the data represent the means (±S.D.).

3. Results

3.1. Titration of the arsenite dose

To study the toxicity of arsenite in zebrafish, we exposed embryos (at 3 days postfertilization [dpf]) to different concentrations of arsenite and examined survival rates at designated time points. As shown in Fig. 1, 93.75 ± 1.25% ($N = 3$) of mock-treated embryos were alive at 120 h after exposure. The survival rates decreased as the time of exposure to and the concentration of (from 0.5 to 50 μM) arsenite increased. At the end of the examination (120 h after exposure), survival rates decreased significantly to 83.33 ± 1.91%, 74.58 ± 2.60%, and 72.5 ± 2.17% ($N = 3$) of embryos treated with 0.5, 5, and 50 μM of arsenite, respectively. Thus, arsenite-induced embryonic death was dose- and time-dependent. Therefore, 50 μM arsenite was selected as the dose for mimicking long-term chronic toxicity in the following experiments.

3.2. Arsenite exposure results in skin lesions that are time- and dose-dependent

We treated the 3 dpf embryos derived from the Tg(k18(2.9):RFP) transgenic line with 5 and 50 μM arsenite and observed the phenotypic defects at specific time points. Although no obvious defects were observed under the light microscope after 48 h of exposure (Fig. 2A), some RFP-positive epithelial cells were distributed abnormally on the trunk when observed under the fluorescent microscope (Fig. 2B, circles). These epithelial cells actually displayed arsenic keratosis (Fig. 2C). In contrast, RFP-positive epithelial cells were distributed evenly on the surface of the embryos from Tg(k18(2.9):RFP) fish without treatment with arsenite (Fig. 2D). The phenotypic defects became more severe with increased time of arsenite exposure. After 72 h of exposure, the keratotic lesions became more evident and the trunk began to twist (Fig. 2E). After 120 h of exposure, more arsenic keratosis appeared on the trunk and the body twist became more severe (Fig. 2F).

We measured the percentage of arsenite-treated fish with skin lesions. No fish with abnormal skin were found after 24 h of exposure to 5 μM arsenite (Fig. 3). As the exposure time increased, the percentage of fish with skin lesion increased: 51.32 ± 2.36%, 65.84 ± 2.46%, 75.65 ± 5.62%, and 86.59 ± 1.65% ($N = 3$) with 48, 72,

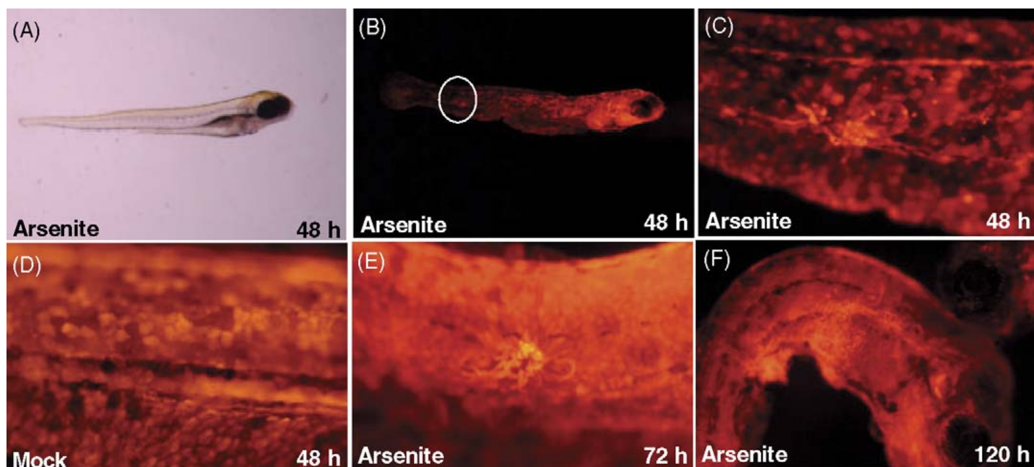

Fig. 2. Arsenite induces toxicity in the transgenic zebrafish. (A) At 48 h of exposure, under light field. (B) The same embryo in (A) but observed under fluorescent field. (C) Enlargement of the circled regions in (B); (D) At 48 h of exposure in water without arsenate (mock control). (E) At 72 h of exposure. (F) At 120 h of exposure.

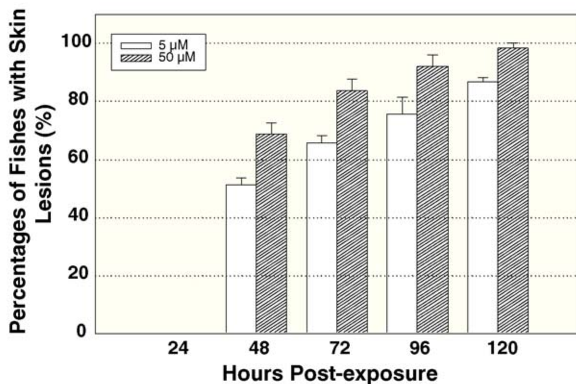

Fig. 3. Arsenite causes skin lesions in a dose-dependent manner. The experiments were performed in triplicate, and the data represent the means (±S.D.).

96, and 120 h of exposure, respectively (Fig. 2A). Similar results were found in the group exposed to 50 μM arsenite (Fig. 3). T-test analysis showed a significant difference between 5 and 50 μM dose groups ($P < 0.01$). However, the F-test revealed no statistical significance between the dose and time of exposure groups ($P > 0.1$), indicating that almost all the fish had skin lesions after long-term arsenite exposure.

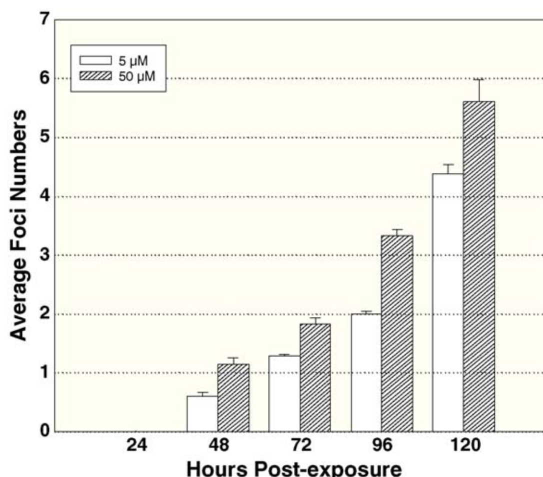

Fig. 4. Arsenite causes skin lesions in fish in a time- and dose-dependent manner. The experiments were performed in triplicate and the data represent the means (±S.D.).

Furthermore, we counted the average number of arsenic keratosis foci in embryos from the Tg(k18(2.9):RFP) line exposed to 5 and 50 μM arsenite. In both groups, no arsenic keratosis had been observed after 24 h of treatment (Fig. 4). However, after 120 h of exposure, the average number of foci was 4.38 ± 0.16 and 5.61 ± 0.36 ($N = 3$) in the 5 and 50 μM arsenite-exposed groups, respectively. At 24–120 h of exposure, the average number of foci increased daily in both the 5 and 50 μM dose groups (Fig. 4). Both T-test (comparing 5 μM with 50 μM dose) and F-test (comparing dose with exposure time) analyses showed statistical significance ($P < 0.01$). On the basis of these observations, we conclude that arsenite exposure results in skin lesions that are time- and dose-dependent.

3.3. Treatment with arsenite results in epithelial cell overproliferation

Long-term arsenite exposure results in skin lesions. One of the most serious effects is malignancy. To confirm whether the arsenic keratosis is due to epithelial cell overproliferation, we used paraffin sections and hematoxylin and eosin staining to check cell proliferation and cell morphology. In the caudal region of a wt section, the superficial stratum is composed of a single cell layer (Fig. 5A). In arsenite-treated embryos, muscle became a loose and wavy structure (compare Fig. 5A with B). Inspection at higher magnification (Fig. 5B) showed that many epithelial cells overproliferated in the caudal region (Fig. 5C, arrowhead).

One advantage of using these Tg(k18(2.9):RFP) transgenic fish embryos to study arsenite toxicity is the epithelial-specific red fluorescence, which makes the observation of dermatological change possible. We can observe the subtle changes of epithelial cells causing skin lesions by monitoring the expression patterns of red fluorescence. After 24 h of exposure, no obvious skin lesion

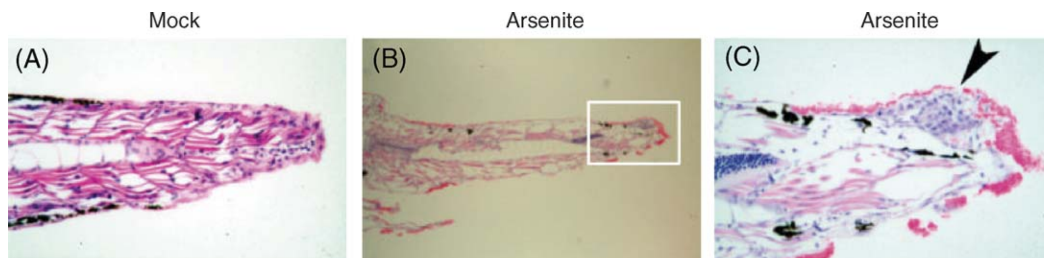

Fig. 5. (A and B) Paraffin sections of the Tg(k18(2.9):RFP) transgenic line after exposure in the water (A) or water containing arsenite (B) for 48 h. (C) Enlargement of the same section at (B); the arrowhead indicates the location of overproliferation of epithelial cells.

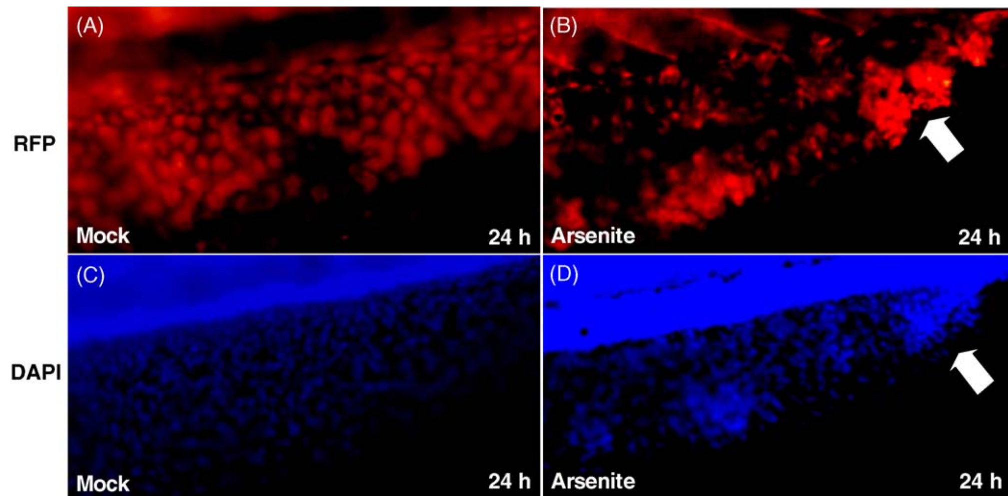

Fig. 6. The 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) staining of the Tg(k18(2.9):RFP) fish line after exposure in water (labeled as Mock) or water containing arsenite (labeled as Arsenite) for 24 h. (A–D) All the pictures are of the same embryos, observed with red fluorescence (A and B) or after DAPI staining (C and D). Arrows in (B) and (D) indicate the location of overproliferation of epithelial cells.

was observed in the anal fin under the light microscope. However, RFP-positive cells form three major colonies (Fig. 6B, arrow) compared to mock-treated embryos (Fig. 6A), suggesting that the epithelial cells overproliferated after arsenite treatment. To confirm this hypothesis, we stained the embryos with DAPI to visualize the nuclei. Results showed that the nuclei of epithelial cells were distributed evenly in the anal fin of the mock-treated embryos (Fig. 6C). In contrast, nuclei of epithelial cells overproliferated at the same region of the anal fin in the arsenite-treated group (Fig. 6B versus Fig. 6C), demonstrating that arsenite exposure is able to induce epithelial cell overproliferation.

4. Discussion

In this study, we take advantage of the transgenic zebrafish, whose red fluorescence is expressed in the stratified epithelia, to study time- and dose-dependent arsenite exposure. We also counted the number of skin foci. Paraffin sectioning combined with DAPI staining experiments were carried out to confirm further that the epithelial cells overproliferated after treatment with arsenite.

4.1. Exposure doses and chronic effects

We used zebrafish as an animal model to study inorganic arsenite-induced toxicity; however, exposure doses and durations are the most important issues that should be addressed. The maximum concentration of arsenic that is permissible by The World Health Organization ranges from 10 to 50 μg/l (10–50 ppb). In an epidemic area, the drinking water contains 160–3400 ppb of arsenic solution. Doses of 5–400 ppm of inorganic arsenic have been used in the mouse model to generate desirable symptoms. The exposure time in mice ranged from 16 weeks to 24 months (Wang et al., 2002). In this study, the concentration of inorganic arsenite ranged from 0.5 to 50 μM (0.0375–3.75 ppm), and the exposure duration was from 2 to 5 days. These doses of arsenite used on zebrafish are reliable and effective. We speculate that this difference in concentration between species is because fish have systemic exposure, whereas, mice have local exposure.

In long-term, low-level arsenic exposure, arsenic tends to concentrate in the ectodermal tissues, including the skin, hair, and nails. As might be expected, the most common lesions resulting from arsenic exposure occur in the skin, and the most striking feature of arsenic-induced lesion is arsenic keratosis. The survival rate was higher than $72.5 \pm 2.17\%$ after treatment with 0.0375–3.75 ppm of inorganic arsenite (Fig. 1). The same arsenite-induced skin lesions were also observed in zebrafish skin after exposure to inorganic arsenite (Fig. 2). On the basis of these observations, we propose that the exposure conditions described in this study can mimic chronic arsenite toxicity.

4.2. Using transgenic animals to study inorganic arsenic-induced toxicity

In mice, inorganic arsenic-induced toxicity is difficult to observe. Previous researchers used transgenic

mice strains to aid in their arsenic studies. These strategies included a tumor copromotion study using Tg.AC (H-ras mutated) transgenic mice, in which skin tumors were generated by coexposure to inorganic arsenite together with 12-*O*-tetradecanoyl phorbol-13-acetate (TPA; Germolec et al., 1998), and studies in which skin tumor was induced more efficiently by exposure to inorganic arsenite in K6/ODC (Chen et al., 2000) and *Folbp2*$^{-/-}$ (Spiegelstein et al., 2005) transgenic mice strains. In this study, we used a transgenic fish line in which skin lesions are easily observable (Fig. 2). In addition, as shown in Fig. 6, the epithelial cells overproliferated after exposure with inorganic arsenite. Thus, treatment with inorganic arsenite of fish embryos from the Tg(k18(2.9):RFP) line may highly facilitate the elucidation of the carcinogenesis mechanism.

4.3. Tg(k18(2.9):RFP) fish may be a useful tool for monitoring environmental changes

Arsenic toxicity leads to multisystem diseases, and the most obvious consequence is skin lesions. Numerous skin changes occur as a result of long-term arsenic exposure. Here, we use the Tg(k18(2.9):RFP) fish line, in which simple epithelial cells and skin epidermis fluoresce red, to monitor arsenite toxicity. The zebrafish k18 gene is expressed both in stratified epithelia (including epidermis) and simple epithelia (Schaffeld et al., 2003; Wang et al., 2006). Because skin is the first physical barrier to protect the body from damage by hazards in the environment, this fish model is a promising way to study arsenite toxicity. In addition, the biggest advantage of using the Tg(k18(2.9):RFP) transgenic line is the red fluorescence. As shown in Fig. 2A, no obvious defects were observed with the light microscope, but some RFP-positive epithelial cells were distributed abnormally on the trunk when viewed with the fluorescence microscope (Fig. 2B, circles), indicating that skin abnormalities are visible after the treatment of Tg(k18(2.9):RFP) fish embryos with arsenite. These observations suggest that the Tg(k18(2.9):RFP) fish line is an excellent model for studying toxicity induced by inorganic arsenite and may have potential for studying other environmental pollutants.

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References

Basu, A., Mahata, J., Gupta, S., Giri, A.K., 2001. Genetic toxicology of a paradoxical human carcinogen, arsenic: a review. Mutat. Res. 488, 171–194.

Chen, Y., Lousis, C.M., Susan, K.G., Sawicki, J.A., O'Brien, T.G., 2000. K6/ODC transgenic mice as a sensitive model for carcinogen identification. Toxicol. Lett. 116, 27–35.

Germolec, D.R., Spalding, J., Yu, H.S., Chen, G.S., Simeonova, P.P., Humble, M.C., Bruccoleri, A., Boorman, G.A., Foley, J.F., Yoshida, T., Luster, M.I., 1998. Arsenic enhancement of skin neoplasia by chronic stimulation of growth factors. Am. J. Pathol. 153, 1775–1785.

Kanisawa, M., Schroeder, H.A., 1969. Life term studies on the effect of trace elements on spontaneous in mice and rats. Cancer Res. 29, 892–895.

Kimmel, C., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development in the zebrafish. Dev. Dyn. 203, 253–310.

Kroes, R., Van Logten, M.J., Berkvens, J.M., De Vries, T., van Esch, G.J., 1974. Study on the carcinogenicity of lead arsenate and sodium arsenate and on the possible synergistic effect of diethylnitrosamine. Food Cosmer. Toxicol. 12, 671–679.

Maharjan, M., Watanabe, C., Ahmad, S.A., Ohtsuka, R., 2005. Arsenic contamination in drinking water and skin manifestations in lowland Nepal: the first community-based survey. Am. J. Trop. Med. Hyg. 73, 477–479.

Morales, K.H., Ryan, L., Kuo, T.L., Wu, M.M., Chen, C.J., 2000. Risk of internal cancers from arsenic in the drinking water. Environ. Health Perspect. 108, 655–661.

Nusslein-Volhard, Dahm, 2002. Zebrafish. Oxford University Press, NY, USA.

Popovicova, J., Moser, G.J., Goldsworthy, T.L., Tice, R.R., 2000. Carcinogenicity and co-carcinogenicity of sodium arsenite in P$^{53+/-}$ male mice. Toxicologist 54, 134.

Ratnaike, R.N., 2003. Acute and chronic arsenic toxicity. Postrad. Med. J. 79, 391–396.

Rossman, T.G., Uddin, A.N., Burns, F.J., Bosland, M.C., 2001. Arsenite is a cocarcinogen with solar ultraviolet radiation for mouse skin: an animal model for arsenic carcinogenesis. Toxicol. Appl. Pharmacol. 176, 64–71.

Rossman, T.G., 2003. Mechanism of arsenic carcinogenesis: an integrated approach. Mutat. Res. 533, 37–65.

Schaffeld, M., Knappe, M., Hunzinger, C., Markl, J., 2003. cDNA sequences of the authentic keratins 8 and 18 in zebrafish. Differentiation 71, 73–82.

Spiegelstein, O., Gould, A., Wlodarczyk, B., Tsie, M., Lu, X., Le, C., Troen, A., Selhub, J., Piedrahita, J.A., Salbaum, M., Kappen, C., Melnyk, S., James, J., Finnell, R.H., 2005. Developmental consequences of in utero sodium arsenate exposure in mice with folate transport deficiencies. Toxicol. Appl. Pharmacol. 203, 18–26.

Tseng, C.H., Huang, Y.K., Huang, Y.L., Chung, C.J., Yang, M.H., Chen, C.J., Hsueh, Y.M., 2005. Arsenic exposure, urinary arsenic speciation, and peripheral vascular disease in blackfoot disease-hyperendemic villages in Taiwan. Toxicol. Appl. Pharmacol. 206, 299–308.

Tucker, R.P., Chiquet-Ehrismann, R., Chevron, M.P., Martin, D., Hall, R.J., Rubin, B.P., 2001. Teneurin-2 is expressed in tissues that reg-

ulate limb and somite pattern formation and is induced in vitro and in situ by FGF8. Dev. Dyn. 220, 27–39.

Waalkes, M.P., Liu, J., Ward, J.M., Diwan, B.A., 2004a. Animal models for arsenic carcinogenesis: inorganic arsenic is a transplacental carcinogen in mice. Toxicol. Appl. Pharmacol. 198, 377–384.

Waalkes, M.P., Ward, J.M., Diwan, B.A., 2004b. 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, 133–141.

Wang, J.P., Qi, L., Moore, M.R., Ng, J.C., 2002. A review of animal models for the study of arsenic carcinogenesis. Toxicol. Lett. 133, 17–31.

Wang, Y.H., Chen, Y.H., Lin, Y.J., Tsai, H.J., 2006. Spatiotemporal expression of zebrafish keratin 18 during early embryogenesis and the establishment of a keratin 18:RFP transgenic line. Gene Expr. Patterns, in press.