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Developmental consequences of in utero sodium arsenate exposure in mice with folate transport deficiencies

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Abstract

Previous studies have demonstrated that mice lacking a functional folate binding protein 2 gene (*Folbp2*^{-/-}) were significantly more sensitive to in utero arsenic exposure than were the wild-type mice similarly exposed. When these mice were fed a folate-deficient diet, the embryotoxic effect of arsenate was further exacerbated. Contrary to expectations, studies on 24-h urinary speciation of sodium arsenate did not demonstrate any significant difference in arsenic biotransformation between *Folbp2*^{-/-} and *Folbp2*^{+/+} mice. To better understand the influence of folate pathway genes on arsenic embryotoxicity, the present investigation utilized transgenic mice with disrupted folate binding protein 1 (*Folbp1*) and reduced folate carrier (*RFC*) genes. Because complete inactivation of *Folbp1* and *RFC* genes results in embryonic lethality, we used heterozygous animals. Overall, no *RFC* genotype-related differences in embryonic susceptibility to arsenic exposure were observed. Embryonic lethality and neural tube defect (NTD) frequency in *Folbp1* mice was dose-dependent and differed from the *RFC* mice; however, no genotype-related differences were observed. The *RFC* heterozygotes tended to have higher plasma levels of S-adenosylhomocysteine (SAH) than did the wild-type controls, although this effect was not robust. It is concluded that genetic modifications at the *Folbp1* and *RFC* loci confers no particular

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sensitivity to arsenic toxicity compared to wild-type controls, thus disproving the working hypothesis that decreased methylating capacity of the genetically modified mice would put them at increased risk for arsenic-induced reproductive toxicity.

Keywords

Arsenic; Teratogenicity; Biotransformation; Detoxification; Folbp1; RFC; Neural tube defects

Introduction

Folic acid deficiency has been shown to be associated with an increased risk for selected birth defects (Nelson, 1960; Nelson et al., 1952; Warkany and Nelson, 1940). Over the past decade, a considerable number of studies have repeatedly demonstrated the protective role of periconceptional folic acid supplementation in preventing neural tube, conotruncal heart, and craniofacial malformations in humans (Shaw et al., 1995a, 1995b, 1995c). Daily supplementation with folic acid at, or in excess of 0.4 mg/ day, has been shown to reduce the incidence of neural tube defects (NTDs) by up to 70% (Berry and Li, 2002; Berry et al., 1999; Czeizel and Dudas, 1992). Despite the accumulating evidence regarding the embryo-protective benefits of periconceptional folic acid supplementation, the mechanism by which this effect is achieved remains unknown.

It is widely accepted that certain human populations are at an increased risk of developing specific birth defects due to genetic predisposition in the form of mutations or polymorphisms in 'developmentally important' genes. Those genes that are involved in folate transport and folate metabolism have been intensively studied in this context; however, thus far, none have emerged as major contributors to susceptibility in all populations (Barber et al., 1998; Rady et al., 2001; Shaw et al., 1998). At the present time, the direct cause of birth defects has been identified for no more than one-third of all cases, and these have been attributed to either purely genetic, or purely environmental, factors (Wilson, 1973). The cause of the remaining proportion of birth defect cases is unknown, but it is widely hypothesized to comprise the interaction of both genetic and environmental components (Finnell et al., 2002a, Khoury et al., 2004; Martin et al., 2003).

Inorganic arsenic (Asi) is a natural element found in the environment (drinking water, air, food) as arsenate [pentavalent, As(V)] or arsenite [trivalent, As(III)] forms. Arsenic has been, and continues to be used as a pesticide, herbicide, and even as a medicament; however, arsenic is mostly known for its ability to elicit toxicity. An association between human exposure to Asi and various forms of cancer has been established for many years (Smith et al., 1992). It has been suspected that Asi is also a human teratogen, and several epidemiological studies have addressed this issue (Shalat et al., 1996). However, in spite of the evidence presented in these studies, a conclusive association between Asi exposure and the occurrence of human malformations has not been established, possibly due to deficits in experimental design (DeSesso, 2001; Holson et al., 2000). Numerous animal studies examined the role of arsenic-induced teratogenicity in various species, different chemical forms, routes of administration, and experimental designs. Although Asi was found to be teratogenic only when administered by injection and not as a result of oral or inhalation exposure, animal studies have consistently shown Asi to induce a specific pattern of malformations, including neural tube and craniofacial defects. In addition, these effects were observed in multiple species with a typical dose-response, indicating that Asi has a direct teratogenic effect (Golub et al., 1998; Holson et al., 2000; Shalat et al., 1996).

Metabolism of As(V) in most mammalian species proceeds via reduction to As(III) and biomethylation to monomethylarsonic acid (MMA(V)), which is then reduced to MMA(III) and biomethylated to dimethylarsinic acid (DMA(V)), which is the major arsenic metabolite excreted in the urine (Vahter and Marafante, 1988). Reduction reactions require glutathione, whereas the biomethylation reactions are catalyzed by methyltransferases and require S-adenosylmethionine (SAM) as the methyl donor (Vahter, 2000; Vahter and Enval, 1983). SAM is eventually regenerated through the homocysteine remethylation cycle, a process that requires 5-methyl-tetrahydrofolate (5M-THF) as a co-factor. Thus, As metabolism is dependant on folate supply, and indeed, it has been shown that methyl deficiency results in alterations of DMA(V) excretion (Vahter and Marafante, 1987). As the pentavalent methylated arsenicals are substantially less toxic than As, any perturbation of arsenic biomethylation, such as that observed under conditions of folic acid deficiency, is hypothesized to increase arsenic-induced toxicity.

The intracellular entry of folates is mediated by folate receptors, which are called folate binding proteins (Folbps) in mice, working in tandem with the reduced folate carrier (RFC). Most FRs/Folbps are externally bound to the plasma membrane by glycosyl-phosphatidylinositol anchors, and have tissue-specific and cell-specific expression patterns (Antony, 1992, 1996). The tissue expression of the *FR-α* isoform is highly specific and is confined to placenta, the brush border membrane of the kidney proximal tubules and to the choroid plexus (Elwood, 1989; Prasad et al., 1994; Selhub and Franklin, 1984). *FR-β*, on the other hand, has a more ubiquitous expression pattern than *FR-α* (Ross et al., 1994). *Folbp1* and *Folbp2*, the mouse homologues of *FR-α* and *FR-β*, have a similar expression pattern (Barber et al., 1999). A recent whole-mount in situ hybridization study in mice demonstrated that *Folbp1* gene is also highly expressed in the yolk sac and in the neural folds just before fusion during neurulation (Saito et al., 2003). *RFC*, on the other hand, is a ubiquitously expressed integral membrane transporter, and has been shown to mediate intestinal folate absorption as well as intracellular entry in numerous tissues (Maddox et al., 2003; Said, 2002; Sirotnak and Tolner, 1999; Wang et al., 2001).

By targeted homologous recombination, we have disrupted the expression of the *Folbp1* and *Folbp2* genes (Piedrahita et al., 1999). While *Folbp2* nullizygous mice (*Folbp2*^{-/-}) develop normally, non-folate-supplemented *Folbp1*^{-/-} embryos are severely developmentally delayed, have multiple structural malformations including neural tube defects, and die by gestational day (E)10.5. However, supplementation of dams with folic acid (25 mg/kg/day) before and throughout gestation is capable of ameliorating the embryolethal effects of the complete loss of *Folbp1* function, and phenotypically rescues the majority of embryos (Finnell et al., 2002b; Tang and Finnell, 2003). In contrast to *Folbp1*^{-/-} mice, the *Folbp1*^{+/-} embryos develop normally and have no apparent pathologies or congenital abnormalities.

Using homologous recombination technologies similar to the methods used to generate the *Folbp1* and *Folbp2* knockout mice, we recently generated mice with an inactivated *RFC* gene (unpublished data). Nullizygous *RFC* embryos (*RFC*^{-/-}) were found to die prematurely in utero; however, in contrast to the *Folbp1*^{-/-} mice, maternal folate supplementation is ineffective in rescuing the lethal phenotype. The *RFC*^{+/-} heterozygous mice develop normally and have no apparent developmental defects.

To test the hypothesis that mice with defective folate transport are more susceptible to arsenical compounds, we have previously treated pregnant *Folbp2*^{-/-} dams with sodium arsenate on E7.5 and 8.5. Surviving fetuses were found to have a higher incidence of NTDs compared to the wild-type mice (Włodarczyk et al., 2001). A folate-deficient diet was found to further exacerbate this phenomenon in the *Folbp2* knockout mice, but was not observed among the wild-type controls. Thus, it was concluded that *Folbp2*^{-/-} mice have an increased

intrinsic susceptibility to in utero arsenic exposure, thereby confirming our hypothesis that defective embryonic folate transport confers enhanced susceptibility to compounds that produce teratogenicity.

In the current study, we wanted to extend our investigation into the role of folate transport and susceptibility of mouse embryos to arsenic exposure during embryonic development. Due to their premature embryonic lethality and complicated phenotypes, neither the *Folbp1*^{-/-} nor *RFC*^{-/-} mice could be used. We therefore examined the susceptibility of *Folbp1*^{+/-} and *RFC*^{+/-} mice to in utero arsenic exposure to determine if impaired methylation capacity of the genetically modified mice explained any observed enhancement of susceptibility to reproductive toxicity. Additionally, we were interested in determining whether arsenic metabolism and excretion is disrupted in the heterozygous RFC mice.

Methods

Inactivation of the reduced folate carrier gene

Cloning of RFC—A bacteriophage λ library (Genome Systems, St. Louis) prepared from the inbred mouse strain 129/Sv genomic DNA was screened by PCR with primers specific for exon 3 of mouse RFC1 (TGCGATACAAGC-CAGTCTTGG and GCACCAGGGAGAATATGTAG-GAGG). We identified one positive clone containing an 80-kb insert. Digestion of the clone with *Hind*III and probing with exon 3 identified a 7.5-kb fragment containing exons 3 and 4 (Fig. 1). This fragment was subcloned into pBluescript (Stratagene) and utilized to develop a creloxP-based conditional knockout targeting construct. The RFC1 clone was restriction mapped and a loxP flanked neo-TK was introduced into the unique *Aoc*I site. An additional loxP site was introduced into the *Nsi*I site located downstream from exon 4 by partial digestion, thus also ensuring that the *Nsi*I site was destroyed. The targeting construct (Fig. 1) was linearized with *Hind*III and introduced into the E14 ES cell line.

Electroporation and validation—The ES cell line, E14TG2a, was utilized and cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum, 0.1 mM 2-mercaptoethanol, and 2 mM glutamine. ES cells were co-cultured with embryonic fibroblast feeders as previously described (Piedrahita et al., 1999). Cells were electroporated essentially as described by Reid et al. (1991), using linearized DNA at a final concentration of 2–5 nM. Electroporated cells were plated at a density of $1\text{--}2 \times 10^6$ cell per 10-cm plate. Twenty-four hours after electroporation, cells were placed in 150 mg/ml of G418. Seven to 10 days later, G418-resistant colonies were selected for expansion and analysis. From 1350 colonies resistant to G418, 162 were analyzed by Southern blot, and four were found to be targeted. Targeted colonies were identified by genomic Southern blotting. As shown in Fig. 1 for the initial targeting event, digestion with *Sac*I, *Xma*I, or *Nde*I, and probing with exon 2 can differentiate between the endogenous and the targeted allele. Once a targeted colony was identified, it was expanded and electroporated with 3 nM of cre-expressing plasmid (pPGK-Cre). Following electroporation, cells were placed in ganciclovir to select for loss of the neo-TK insert. Of 60 clones analyzed, 33 had lost the neo-TK, but not exon 3 and 4, while 27 clones had lost both the neo-TK insert and exons 3–4. The latter colonies were further expanded and used to generate germ line chimeras. As shown in Fig. 1C, the deletion event resulted in the complete removal of exons 3 and 4 of the RFC gene. This deleted allele was differentiated from the endogenous allele using an *Apa*I digest and probing with exon 2.

RFC chimera generation—We generated chimeras as previously described (Piedrahita et al., 1992). Animals considered to be chimeric based on coat color were mated with inbred

C57BL/6J dams. DNA samples were isolated from the tails of the ES cell-derived animals using the Puregene reagents (Gentra Systems, Minneapolis, MN), and were analyzed for the presence of disrupted RFC1 gene by Southern blot analysis. Germline chimeras were identified and heterozygous offspring were maintained on the hybrid stock genetic background into adulthood using brother–sister matings to generate homozygous mutants.

Arsenic teratogenicity study

All mice were housed in clear polycarbonate micro-isolator cages, allowed free access to water and food, and were maintained on a 12-h light/dark cycle in the Vivarium at the Institute of Biosciences and Technology in Houston, TX. The mice were maintained on Harlan Sani-chips bedding (Harlan Teklad, Madison, WI) to minimize vitamin intake from bedding consumption. The institutional animal care and use committee approved this study. Virgin *Folbp1*^{+/-} females were mated overnight with *Folbp1*^{+/+} males, and examined for the presence of a vaginal plug the following morning. The onset of gestation was set at 10 p.m. of the previous night, the midpoint of the dark cycle (Snell et al., 1948). On E7.5 and 8.5, pregnant dams received an intraperitoneal (i.p.) injection (10 µl/g body weight) of sodium arsenate (Sigma Chemicals, St. Louis, MO) at a dose of 30, 35, or 40 mg/kg. These dosages are in excess of any human exposure to inorganic arsenic reported, but are consistent with our previously published efforts using this model system (Spiegelstein et al., 2003a; Wlodarczyk et al., 2001). The compound was injected intraperitoneally to ensure more control over the delivery and recovery of the compound in the urinary excretion studies. Experiments in the *RFC* mice were performed in a manner similar to that described above for the *Folbp1* mice. In these experiments, *RFC*^{+/+} dams were mated with *RFC*^{+/-} sires and the reciprocal cross (*RFC*^{+/-} dams and *RFC*^{+/+} sires) was also conducted. The dams received 40 mg/kg arsenate. Control mice were given intraperitoneal injections of sterile water USP (Abbott Laboratories, Chicago, IL), at a dose volume of 10 µl/g body weight on the same gestational days. On E18.5, the *Folbp1* and *RFC* dams were sacrificed by cervical dislocation, the abdomen opened and the gravid uteri removed. The location of all viable fetuses and resorptions were recorded, and the fetuses were examined for the presence of gross morphological abnormalities. The tail of each fetus was removed and processed to determine their folate transport allele genotypes.

Genotyping

Genotyping for the *Folbp1* mutant allele was performed using standard protocols with the following primers (5'–3'): mutant forward—ATC GCC TTC TAT CGC CTT CTT GA; mutant reverse—TGC ATT CCG ATG TCA TAG TTC CG; wild-type forward—AAG TGC AAG GCT GCA TGT GG; wild-type reverse—CAT TCC GAT GTC ATA GTT CCG C. The PCR conditions were an initial denaturation at 95°C for 5 min, followed by 30 cycles of annealing (60°C for 1 min), extension (72°C for 2 min), denaturation (95°C for 1 min) and a final extension at 72°C for 10 min in an automated thermocycler (Biometra, Goettingen, Germany). The 179-bp or 1.2-kb products corresponded to (+/+) and (-/-) genotype, respectively.

Genotyping for the *RFC* mutant allele was similarly performed with the following primers (5'–3'): mutant forward—GAT TCC AAG CAT GTC CAC TAC CC; mutant reverse—CAG AAG GCA AGT GTC TGT ATG TG; wild-type forward—AAG GCA ATG CAG GCA GAT ACG TGG; wild-type reverse—GCT CTG TCC TTA TAG GGG TTG TGA. The PCR conditions were an initial denaturation at 95°C for 5 min, followed by 30 cycles of annealing (55°C for 30 s), extension (72°C for 30 s), denaturation (95°C for 30 s), and a final extension at 72°C for 10 min in an automated thermocycler (Biometra). The 105- or 350-bp products corresponded to (+/+) and (-/-) genotype, respectively.

Arsenic speciation study

Detailed descriptions of the methods used have been previously described (Spiegelstein et al., 2003a). Briefly, in the initial phase of the study under conditions of normal folate intake, six *RFC*^{+/+} and six *RFC*^{+/-} adult mice, 2–5 months of age, were placed on an amino acid defined diet containing 2.7 mg folate/kg diet and 1% succinyl sulfathiazole (control diet; Dyets #517839, Bethlehem, PA) for approximately 20 days. Each mouse received a single 1 mg/kg intraperitoneal (i.p.) injection of sodium arsenate (Sigma) dissolved in sterile water (Abbott Laboratories, Abbott Park, IL), and were placed individually in a metabolic cage (Nalge Nunc International, Rochester, NY). Urine collected over a period of 24 h post-injection was stored at –80 °C until analyzed. At the completion of the first phase of the study, mice were placed on an amino acid defined diet containing 0.3 mg folate/kg diet (reduced folate diet; Dyets #517796, Bethlehem, PA) for approximately 25 days. The composition of the reduced folate diet was exactly the same as the control diet, with the exception of its folate content. Urine was collected in a similar manner.

Urinary arsenic analysis

Detailed descriptions of the methods used have been previously described (Spiegelstein et al., 2003a). Analysis was performed by ion pair chromatographic separation and anion exchange chromatographic separation with hydride generation atomic fluorescence detection (Le et al., 2000). The following limits of detection were obtained: 0.5 µg/l for As(III) and MMA(V); 1 µg/l for DMA(V) and As(V); 2 µg/l for MMA(III) and DMA(III); 7 µg/l for trimethylarsine oxide. The presence of MMA(III), DMA(III), and trimethylarsine oxide was not detected in all urine samples.

Folate and homocysteine metabolites analysis in plasma

Total folate levels in plasma were determined using the *Lactobacillus casei* microbiological assay as previously described (Spiegelstein et al., 2003a). Analysis of SAH and SAM was performed using HPLC with electrochemical detection, as previously described (Melnik et al., 2000).

Statistical analysis

Teratogenicity data was analyzed for statistical significance using the Chi-squared test. Genotype-specific analysis for statistical significance in terms of plasma folate, SAM, SAH and adenosine concentrations, the SAM/SAH ratios, and the urinary excretion of arsenicals was performed using Student's *t* test. Dietary-specific effects were analyzed using a paired *t* test. In all comparisons, significance was set at a *P* value of 0.05, two sided.

Results

The embryotoxicity and teratogenicity of arsenic in the *RFC* knockout mice is presented in Table 1. Among the control *RFC* treatment groups, we did not observe any fetuses with NTDs. Genotyping the surviving fetuses revealed that there were equal numbers of *RFC*^{+/-} and *RFC*^{+/+} fetuses present. Administration of 40 mg/kg sodium arsenate resulted in embryonic lethality frequencies similar to those observed for the controls. The percentages of liveborn fetuses between the two genotypes were indistinguishable one from the other (Table 1). In addition, no significant differences in the rates of exencephaly among the *RFC*^{+/-} and *RFC*^{+/+} fetuses were observed. Collectively, no *RFC* genotype-related differences in embryonic susceptibility to arsenic exposure were observed.

The developmental outcome of in utero arsenic exposure in the *Folbp1* knockout mice is presented in Table 2. In all arsenic-treated groups of *Folbp1* mice, there was a significantly

higher rate of resorptions and NTDs when compared to the control mice. There were no fetuses with NTDs in the *Folbp1* control group, and the rate of resorptions was quite low (6.3%). Administration of 30 mg/kg sodium arsenate resulted in 45% embryo lethality, and the number of term *Folbp1*^{+/+} and *Folbp1*^{+/-} fetuses were similar. Among these fetuses, 11–18% were exencephalic, which was not significantly different between the genotypes (Table 1). Administration of 35 mg/kg arsenic increased the rate of embryonic lethality to 62%, and the rate of NTDs was increased to 35–36%, which was significantly higher than the NTD response frequency following arsenate treatment at 30 mg/kg. When dams were injected with 40 mg/kg arsenic, 82% of embryos were resorbed. Overall, no statistically significant *Folbp1* genotype related differences in occurrence of NTDs were detected.

Plasma folate, SAM, and SAH concentrations

The total folate levels in the plasma of the *RFC* knockout mice are presented in Table 3. During the initial phase of the study while the dams were maintained on a control diet, plasma folate levels were approximately 35 ng/ml, and did not differ between the *RFC*^{+/+} and *RFC*^{+/-} mice. Folate levels dropped significantly by sevenfold to ninefold during the reduced dietary folate phase in both *RFC* genotypes, indicating that the reduction of dietary folate content from 2.7 to 0.3 mg/kg diet was sufficient for inducing a mild folate deficiency, irrespective of the genotype.

The plasma concentrations of SAM and SAH, as well as the SAM/SAH ratio of the *RFC* knockout mice, are presented in Table 3. Statistically significant differences between the two genotypes were observed only for SAH levels during folate deficiency, when the *RFC*^{+/-} mice had higher levels than the *RFC*^{+/+} mice (64 vs. 39 pmol/ml, respectively). A similar trend in the genotype-related differences was also observed during the control phase; however, it was not statistically significant.

Urinary arsenic speciation

The 24-h urinary speciation data in *RFC* mice is presented in Table 4. Overall, DMA(V) was the primary arsenic species excreted, accounting for 41–43% of the administered dose during the control diet and 35% during folate deficiency. Excretion of As(V), the parent compound, accounted for 25–27% of the administered dose among animals fed the control diet, and 19–24% during the dietary induced folate deficiency. The average total amount of arsenicals excreted in the urine was 73–77% of the administered dose during the control diet, and 58–63% during folate deficiency. As(III) was excreted in very small amounts (4–6%), whereas the presence of MMA(III), DMA(III) and trimethylarsine oxide was not detected. Genotype-specific differences were observed in the excretion of As(V) during the folate-deficient diet between *RFC*^{+/+} and *RFC*^{+/-} mice. Statistically significant dietary effects were observed only for total arsenic excretion in both *RFC*^{+/+} and *RFC*^{+/-} mice.

Discussion

Complete loss of *Folbp1* function has been shown to be embryolethal due to the importance of this gene product in normal murine embryonic development (Piedrahita et al., 1999). Amelioration of the majority of lethality and structural malformations associated with the *Folbp1* nullizygous genotype has thus far been attained by providing high doses of folates (>12.5 mg/kg, body weight) to pregnant dams, before and throughout gestation (Finnell et al., 2002b; Spiegelstein et al., in press; Tang and Finnell, 2003). Among the *Folbp1*^{-/-} mice that have been rescued, a consistent proportion of mice present with neural tube, ocular, body wall, craniofacial and cardiac malformations. Those mice that are homozygous for the mutant *RFC* allele die during early development, most often by E9.5 (unpublished data). However, unlike the *Folbp1*-deficient mice, we failed to rescue *RFC*^{-/-} embryos by

supplementing dams with high doses of various folates. The *RFC* heterozygotes do not appear to have any obvious adverse health problems. Being cognizant that *Folbp1*^{-/-} and *RFC*^{-/-} homozygous knockouts die in early embryogenesis, we used heterozygous to wild-type matings, where wild-type and heterozygous offspring are produced in a 1:1 ratio, thereby providing both experimental and control fetuses. In addition, the heterozygous genotype more closely resembles the situation in the human population, where gene mutations and polymorphisms exist far more frequently in the heterozygous state rather than as homozygotes.

In the *RFC* knockout mice, regardless of the treatment or mating strategy used, no significant differences in frequencies of embryonic lethality were observed. Embryonic lethality was not higher in the mutant *RFC*^{+/-} offspring compared to the wild type, as evident by the equal distribution of heterozygous and wild-type liveborn pups. Among the control mice, while no NTDs were observed, there was an overall NTD response frequency of approximately 20% among *RFC* arsenic-treated fetuses. No significant maternal genotype effect was observed, and, moreover, within each arsenic treatment group, no significant differences between the two genotypes were observed. Collectively, we can conclude that in our model system, the heterozygous *RFC* allele confers no additional susceptibility to the development of NTDs secondary to an in utero arsenic exposure.

In contrast to the *RFC* mice, *Folbp1* mice were highly susceptible to arsenic exposure, as evidenced by the significantly high and dose-related frequency of embryonic lethality. These rates were similar to what was previously observed with arsenic treatment in *Folbp2* mice (Włodarczyk et al., 2001). Although the percentage of exencephalic fetuses was dose dependent, the absolute numbers were very low and did not permit testing for statistical significance or genotype-related differences in susceptibility. In the *Folbp2* nullizygous mice, a clear susceptibility profile to in utero arsenic exposure was observed, which was not present in these mice when treated with valproic acid, another well-known NTD-inducing teratogen (Spiegelstein et al., 2003b).

Although the reduced folate carrier acts in concert with folate binding proteins to transport folate molecule into cells, and the total inactivation of the *RFC* gene is embryolethal, the offspring of heterozygous *RFC* dams were much less susceptible to arsenic than were either the *Folbp1*^{+/-} or *Folbp2*^{-/-} mice. One possible explanation rests on the differences in the genetic backgrounds of these different transgenic mice. The *Folbp1* and *Folbp2* knockout mice were originally backcrossed to the highly inbred C57BL/6J strain, whereas the *RFC* null allele was transferred to the SWV/Fnn inbred strain. The teratological literature is replete with studies demonstrating that inbred strains of mice respond differently to the same teratogenic insult.

Heterozygosity for the null allele at the *RFC* locus appears to have no adverse effects on steady state plasma folate and homocysteine remethylation cycle metabolites (Table 3). The lack of effect is evident under both folate replete and depleted conditions. Assessing the full effect of the *RFC* gene inactivation is not possible, due to its homozygous lethal phenotype. The impact on folate concentrations is similar to what is observed with *Folbp1* heterozygous mice (Spiegelstein et al., 2003a). As would be expected when there are no apparent effects of *RFC* heterozygosity on folate and homocysteine metabolites, the detoxification of inorganic arsenic was similarly unaffected. However, much like our previous observations, the folate-deficient diet resulted in reduced total arsenic excretion (Spiegelstein et al., 2003a).

In conclusion, heterozygosity for both the *RFC* and *Folbp1* genes did not appear to adversely affect the sensitivity of embryos to sodium arsenate, an NTD-inducing toxicant

during neurulation, thus disproving our original hypothesis. The differential susceptibility profile between the two knockout mouse strains is likely the result of differences in their genetic background, rather than defective folate transport. In addition, heterozygous adult *RFC* mice did not seem to have any alteration on plasma folate levels or circulating homocysteine remethylation cycle metabolite levels, nor was arsenic biotransformation affected.

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Fig. 1.
Targeted inactivation of the murine RFC gene. (A) Endogenous genomic loci. (B) Targeted RFC loci before Cre-mediated excision. Heavy line represents targeting construct; arrow head represents the location of loxP sites. (C) Targeted loci following Cre-mediated excision.

Table 1

Developmental outcome following in utero arsenic exposure in *RFC* knockout mice

Dam genotype	Sire genotype	Dose (mg/kg)	No. of litters (N)	No. of implants (N)	No. of resorptions (%) ^a	No. of NTDs (%) ^b	Liveborn fetuses no. (%) ^b		Fetuses with NTDs no. (%) ^c	
							<i>RFC</i> ^{+/+}	<i>RFC</i> ^{+/-}	<i>RFC</i> ^{+/+}	<i>RFC</i> ^{+/-}
<i>RFC</i> ^{+/+}	<i>RFC</i> ^{+/-}	0	8	96	11 (11.5)	0	38 (44.7)	47 (55.3)	0	0
<i>RFC</i> ^{+/-}	<i>RFC</i> ^{+/+}		10	153	39 (25.5)	0	63 (55.3)	51 (44.7)	0	0
<i>RFC</i> ^{+/+}	<i>RFC</i> ^{+/-}	40	20	250	55 (22.0)	31* (18.9)	81 (49.4)	83 (50.6)	12 (12.9)	19 (18.6)
<i>RFC</i> ^{+/-}	<i>RFC</i> ^{+/+}		13	156	19 (12.2)	25* (22.3)	59 (52.7)	53 (47.3)	15 (20.3)	10 (15.9)

^a Percent from the total number of implants.

^b Percent from the total number of liveborn fetuses (*RFC*^{+/+} and *RFC*^{+/-} combined).

^c Percent from the liveborn fetuses of the same genotype.

* Significantly different than the respective controls ($P < 0.05$).

Table 2

Developmental outcome following in utero arsenic exposure in *Folbp1* knockout mice

Dam	Sire	Dose (mg/kg)	No. of litters (N)	No. of implants (N)	No. of resorptions (%) ^a	No. of NTDs (%) ^b	Liveborn fetuses no. (%) ^b		Fetuses with NTDs no. (%) ^c	
							<i>Folbp1</i> ^{+/+}	<i>Folbp1</i> ^{+/-}	<i>Folbp1</i> ^{+/+}	<i>Folbp1</i> ^{+/-}
<i>Folbp1</i> ^{+/-}	<i>Folbp1</i> ^{+/+}	0	11	95	6 (6.3)	0	48 (53.9)	41 (46.1)	0	0
		30	8	73	33 (45.2) *	6 (15.0) *	18 (45.0)	22 (55.0)	2 (11.1)	4 (18.2)
		35	12	105	65 (61.9) *	14 (35.9) **, *	22 (56.4)	17 (43.6)	8 (36.4)	6 (35.3)
		40	12	111	91 (82.0) **, *	5 (26.3) *	11 (57.9)	8 (42.1)	4 (36.4)	1 (12.5)

^a Percent of implants.

^b Percent from the total number of liveborn fetuses (*Folbp1*^{+/+} and *Folbp1*^{+/-} combined).

^c Percent from the liveborn fetuses of the same genotype.

* Significantly different compared to vehicle treated controls ($P < 0.001$).

Table 3

SAM and SAH plasma concentrations and the SAM/SAH ratios in *RFC* knockout mice

Diet	Genotype	Folate (ng/ml)	SAH ^a (pmol/ml)	SAM ^a (pmol/ml)	SAM/SAH ^a
Control diet ^b	<i>RFC</i> ^{+/+}	35.6 ± 2.5	39.1 ± 8.3	150.2 ± 20.3	3.9 ± 0.8
	<i>RFC</i> ^{-/-}	34.4 ± 6.3	54.1 ± 17.5	159.4 ± 34.9	3.1 ± 0.9
Reduced folate ^b	<i>RFC</i> ^{+/+}	4.2 ± 1.0**	41.7 ± 9.9	152.5 ± 52.6	3.9 ± 1.6
	<i>RFC</i> ^{-/-}	5.0 ± 1.5**	63.6 ± 21.9*	147.0 ± 33.3	2.6 ± 1.0

^aValues are expressed as the mean ± standard deviation.

^bControl diet: folate content 2.7 mg/kg diet. Reduced folate diet: folate content 0.3 mg/kg diet.

* Genotype effect: statistically significant compared to the *RFC*^{+/+} mice on the same diet. Student's *t* test, *P* < 0.05, two-tailed.

** Dietary effect: statistically significant compared to the same genotype maintained on the control diet. Paired *t* test, *P* < 0.05, two tailed.

Table 4

Twenty-four hour urinary speciation of arsenicals in *RFC* knockout mice

Diet ^a	Mice genotype	As(V) ^b (%)	As(III) ^b (%)	MMA(V) ^b (%)	DMA(V) ^b (%)	Total ^b (%)
Control	<i>RFC</i> ^{+/+}	25.4 ± 14.9	6.2 ± 4.2	0.42 ± 0.32	40.9 ± 10.2	72.9 ± 2.2
	<i>RFC</i> ^{-/-}	27.1 ± 14.3	6.0 ± 5.1	0.41 ± 0.35	43.1 ± 7.7	76.6 ± 4.5
Reduced folate	<i>RFC</i> ^{+/+}	18.5 ± 3.9	4.2 ± 2.0	ND ^c	35.1 ± 5.5	57.8 ± 7.5*
	<i>RFC</i> ^{-/-}	23.6 ± 3.5	4.1 ± 1.3	ND ^c	35.3 ± 6.4	63.1 ± 9.6*

^aControl diet: 2.7 mg folate /kg diet; reduced folate diet: 0.3 mg folate /kg diet.

^bValues are expressed as the mean ± standard deviation percentage of the administered dose. Arsenate, As(V); arsenite, As(III); monomethylarsonic acid, MMA(V); dimethylarsonic acid, DMA(V).

^cNot detected: during folate deficiency, MMA(V) was not detected in the urine of 5/6 *RFC*^{+/-} and 6/6 *RFC*^{+/+} mice.

* Dietary effect: statistically significant compared to the same genotype maintained on the control diet. Paired *t* test, *P* < 0.05, two-tailed.