Toxic metals are ubiquitous in the environment and are known to cause detrimental health effects. Exposure to toxic metals during the prenatal period is of particular concern, and exposure can occur as a result of diet, drinking water, airborne particles, consumer products, and certain occupational environments (Tabacova 1986). Evidence suggests that many toxic metals indeed cross the placental barrier (Tabacova 1986). Further, toxic metal exposure can result in adverse pregnancy outcomes such as spontaneous abortion, stillbirth, preterm birth (Ahmad et al. 2001), and congenital malformations (Kwok et al. 2006; Zierler et al. 1988). Clearly, there is public health concern surrounding environmental exposure–mediated birth defects, which is supported by both epidemiological and animal-based evidence. Still, the underlying pathophysiological mechanisms linking prenatal exposures to developmental disorders remain largely unknown.

The focus of the present study was to select seven high-priority toxic metals and/or metalloids on the basis of their presence in the environment and their known or suspected roles as developmental toxicants. The following metals were selected for study: cadmium (Cd), chromium (Cr), inorganic arsenic (iAs), lead (Pb), mercury (Hg), nickel (Ni), and selenium (Se). Four of these—Cd, Hg, iAs, and Pb—are ranked in the top 10 most hazardous substances by the Agency for Toxic Substances and Disease Registry (ATSDR 2010). Several of these metals have been associated with structural malformations and/or neural tube defects in animal models (Chaineau et al. 1990; Fernandez et al. 2004; Gilani and Marano 1980; Gruenwald 1958; Hovland et al. 1999; Messele and Webster 1982; Tabacova et al. 1996; Thompson and Bannigan 2001). In addition to the toxicological data, several epidemiological studies have also examined the relationship between metal exposure and early life outcomes in infants. For example, prenatal exposure to iAs during drinking water has been associated with adverse pregnancy outcomes such as spontaneous abortion, stillbirth, preterm birth (Ahmad et al. 2001), and congenital malformations (Kwok et al. 2006; Zierler et al. 1988).

Conclusions: Our findings highlight a novel approach to computationally identify a targeted biological pathway for examining birth defects prevention.
1990), which served as a positive control for the in ovo experiments.

**Identifying metal-associated genes with roles in development.** Once metal-associated genes/proteins were identified using the CTD database, we performed biological function enrichment analysis using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA). Specifically, genes with known involvement in embryonic development and developmental disorders were identified and referred to as ‘development’ associated.

**Predicting pathways involved in metal-induced developmental disorders.** Molecular networks related to metal-associated genes involved in development were identified using IPA. This knowledge database provides a collection of gene-to-phenotype associations, molecular interactions, regulatory events, and chemical knowledge accumulated to develop a global molecular network. In IPA, metal-associated genes were mapped to their global molecular networks, and networks integrating proteins encoded by the metal- and development-associated genes were algorithmically generated based on their connectivity. Pathway enrichment analysis was performed to identify canonical pathways significantly associated with constructed networks. Statistical significance of each constructed network was evaluated using Fisher’s exact test.

**In ovo whole chick embryo culture.** The most significant canonical pathway identified through network analysis was ranked and validated for its involvement in embryonic development using the chick embryo model. Specifically, we used in ovo whole chick embryo culture assay, a well-established model for teratogenicity assessment (Kucera et al. 1993), to test the computational prediction that the GR signaling pathway is involved in metal-induced developmental disorders. All experimental procedures were conducted on embryos < 10 days of age and thus were exempt from oversight by the University of North Carolina Institutional Animal Care and Use Committee. We obtained fertilized white leghorn chicken eggs from Charles River Laboratories (North Franklin, CT, USA). Eggs were randomly selected and divided into seven different treatment groups immediately before incubation. The treatment groups were as follows: control [phosphate-buffered saline (PBS) only]; vehicle control (0.1% ethanol); phenytoin, a positive control for neural tube defects (Fisher Scientific); iAs as sodium arsenite (iAs³⁺; Sigma-Aldrich, St. Louis, MO); cortisolone (0.1 µM, a GR inhibitor) (Fisher Scientific); phenytoin plus cortisolone; and iAs³⁺ plus cortisolone. We selected the concentration of cortisolone on the basis of previous studies (Harlow et al. 1987; Turrell et al. 1974).

Ten to 12 embryos were examined per treatment group in four independent biological replicates. Eggs were incubated at 100°F at a relative humidity of 55%, with the day on which the eggs were incubated counted as day 0. The eggs were dosed on day 3 of incubation. First, the eggs were swabbed with 70% ethanol and the blunt end of the egg was stuck with forceps to make a small hole, following an established protocol (Kucera et al. 1993; Menon and Pratten 2009). Eggs were injected with 100 µL of either PBS alone, vehicle (0.1% ethanol), phenytoin (400 µM), iAs³⁺ (0.1 µM, or 7.5 ppb), cortisolone (2 µM), phenytoin plus cortisolone, or iAs³⁺ plus cortisolone, and then incubated until day 6. On day 6, embryos were removed and analyzed for gross malformations and scored according to established morphological scoring criteria (described by Menon and Pratten 2009) [see Supplemental Material, Table S1 (http://dx.doi.org/10.1289/ehp.1205659)]. Only viable embryos, defined by the presence of a beating heart and visibly circulating blood in the embryo and yolky sac, were included and assigned morphological scores. Crown–rump length was taken as a measure of embryo growth.

Data were analyzed using Graphpad Prism 5 software (Graphpad Software Inc., San Diego, CA). To assess the morphological scoring criteria (vitelline circulation, flexion, heart, brain, gross facial deformities, and limbs) between exposure groups, we used Kruskal-Wallis one-way analysis of variance (ANOVA) followed by Mann-Whitney U-test for non-parametric data. The statistical process was carried out as described by Hewitt et al. (2005). The means ± SEs of the morphological scores were determined for the four independent experiments. Embryo growth, as measured by crown–rump length, was analyzed using parametric statistical tests (ANOVA followed by Dunnnett’s post hoc test). Statistical significance was set at p < 0.05.

**Quantitative reverse-transcription polymerase chain reaction (qRT-PCR).** We assessed the gene expression levels of two transcription factors, NF-kB1 (nuclear factor kappa B1) and AP1 (activating protein 1). AP1 comprises c-FOS and c-JUN, and these genes were assessed relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using qRT-PCR; primer sequences are shown in Supplemental Material, Table S2 (http://dx.doi.org/10.1289/ehp.1205659). Total RNA was isolated from the whole head regions of treated and untreated embryos (n = 3 embryos/group) using an RNaseasy® Mini Kit (QIAGEN, Valencia, CA). RNA was quantified with the NanoDrop® 1000 Spectrophotometer (Thermo Scientific, Waltham, MA), and integrity was verified with a model 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA). Fold changes between the treatment group and the control group were calculated using delta-delta cycle threshold (ΔΔCt) values and normalized with GAPDH as a housekeeping gene. Statistical significance of the transcript levels between treatment groups versus the control group was calculated using an unpaired t-test.

**Chick embryo midbrain micromass (MM) culture in vitro assay.** We further evaluated the role of the GR pathway as a mediator of metal-induced neurodevelopmental toxicity using the in vitro chick embryo MM culture assay. The midbrain MM culture assay is a well-accepted method to screen for developmental toxicity of compounds, and was performed according to previously established protocols (Flinn and Orton 1984; L’Huillier et al. 2002). Midbrains were removed from chick embryos on day 6 and trypsinized to prepare single-cell suspensions in Ham’s F12 culture medium containing 10% fetal calf serum, 200 mM L-glutamine, penicillin (50 units/mL), and streptomycin (50 µg/mL). Cell density was estimated using a hemocytometer and adjusted to 1 × 10⁴ cells/mL. A total of 10 µL of the cell suspension was pipetted into the center of a collagen-coated well in a 96-well culture plate. After 24 hr, midbrain MM cultures were treated with iAs³⁺ (1, 2, or 5 µM), Cd as CdCl₂ (1, 2, 5, or 8 µM), phenytoin (100 µM), cortisolone (2 µM), cortisolone plus iAs³⁺, cortisolone plus CdCl₂, cortisolone plus phenytoin. The assay included positive and negative controls for cytotoxicity, namely, 5-fluorouracil (5-Fu) and penicillin G (PenG), respectively. Midbrain MM culture cytotoxicity was measured after 5 days in culture using the one-step resazurin reduction assay (O’Brien et al. 2000). Optical density was measured on a Synergy™ HT Multi-Mode Microplate Reader (BioTek, Winooski, VT), with an excitation filter 530 ± 25 nm and emission filter 590 ± 35 nm indicating cell viability and metabolic activity. Statistical analysis was performed using Graphpad Prism 5 software. We used Student’s t-test for comparison between two groups and one-way ANOVA followed by an appropriate post hoc test (e.g., Dunnett or Bonferroni) for comparisons among groups. A p-value < 0.05 was considered statistically significant.

**Results**

We used a novel approach to identify biological pathways that may mediate environmentally induced birth defects. Specifically, our strategy involved using the CTD to identify genes associated with environmental metals, filtering these genes for biological function related to birth defects and development, and examining the genes for known biological interactions in the cell [see Supplemental Material, Figure S1 (http://dx.doi.org/10.1289/ehp.1205659)].

**Metal- and development-associated genes identified.** The in silico approach first involved the identification of genes associated with seven metals: Cd, Cr, Hg, iAs, Ni, Pb, and Se. Genes with known association to the metals...
of interest were identified using the CTD [see Supplemental Material, Table S3 (http://dx.doi.org/10.1289/ehp.1205659)]. The CTD contains data on broad relationships between genes and environmental toxicants (we queried for metals), known effects of toxicants on gene expression, and genes encoding proteins that have altered metal-associated changes in protein abundance and protein activity. Thus, all genes identified had a known relationship with at least one of the seven metals: Cd (n = 518 genes), Cr (n = 175 genes), Hg (n = 334 genes), iAs (n = 1,880 genes), Ni (n = 637 genes), Pb (n = 774 genes), and Se (n = 1,616 genes) (see Supplemental Material, Tables S3 and S4). In addition, genes associated with phenytoin, a known teratogen, were identified as a positive control (n = 138) (see Supplemental Material, Tables S3 and S4).

The lists of metal-associated genes were subsequently filtered for genes/proteins with known relationships to organismal development and birth defects. Specifically, we performed biological function enrichment analysis; genes related to either “embryonic development” or “developmental disorders” were identified and extracted from the lists. The resultant numbers of genes that were identified as both metal- and development-associated ranged from 76 for Cr to 604 for iAs (see Supplemental Material, Tables S3 and S5 (http://dx.doi.org/10.1289/ehp.1205659)).

Predicting pathways influencing metal-induced developmental disorders. Biological pathways that were enriched among the metal- and development-associated genes were algorithmically constructed. For each of the metals and for phenytoin, we selected the highest ranking (e.g., top five) canonical pathways. Because of some overlap, this resulted in a total of 22 unique canonical pathways [Figure 1; for a complete list of all top-ranking pathways and their p-values, see Supplemental, Table S6 (http://dx.doi.org/10.1289/ehp.1205659)].

The pathway analysis revealed that, in general, distinct pathways were highly enriched among genes associated with each of the metals (Figure 1). Still, many of the pathways were enriched between two or more metals. The GR signaling pathway was a high ranking canonical pathway for Cd, Hg, iAs, and Se. Notably, this indicates that genes associated with these four metal- and development-associated genes commonly mapped to the GR signaling pathway. Networks associated with phenytoin, a known teratogen, also showed significant enrichment for biological pathways that overlapped with those associated with metal exposure, including the GR signaling pathway. Other pathways of interest were identified as being related to the metal- and development-associated genes, such as the aryl hydrocarbon receptor (AHR) pathway identified for Cr and phenytoin (Figure 1).

To further establish environmental exposure–mediated network interactions, we performed a subsequent analysis using a combined list of all the metal- and development-associated genes together (n = 855 genes). Within the resulting metal-associated networks, the GR signaling pathway was the most significantly (p < 10⁻⁶) enriched pathway [see Supplemental Material, Figure S2 (http://dx.doi.org/10.1289/ehp.1205659)]. Many of the genes that encode proteins involved in the canonical GR signaling pathway have known association with numerous metals as defined in the CTD. For example, NF-κB1, a protein involved in the GR signaling pathway, has a known relationship to six of the seven metals (CTD 2011). Likewise, AP1 (composed of c-FOS and c-JUN) and c-JUN N-terminal kinase (JNK) are also associated with multiple metals (CTD 2011) (see Supplemental Material, Figure S2).

Validation of computational prediction by the embryo culture assay. Given the computational analysis predicting association of Cd, Hg, iAs, and Se with the GR signaling pathway, we set out to validate the findings using an in ovo whole chick embryo culture assay. Our hypothesis was that the GR mediates metal-induced developmental defects. iAs was prioritized for testing, and results were compared to those of phenytoin, a GR-dependent teratogen (Kay et al. 1990). A total of 10–12 embryos per treatment group were examined in four independent biological replicates.

Phenytoin, the positive teratogenic control, induced malformations in 56% of the embryos, and iAs induced malformations in 59% (Table 1). No phenotypic abnormalities were observed in embryos treated with PBS or the vehicle control (Figure 2A, Table 1). For phenytoin and iAs, observed morphological defects included abnormalities in the head fold region, microcephaly, anterior neural tube defects, and gross facial deformities (Figure 2B,C). The craniofacial defects included altered optic and neck development and malformed facial arches.

The in ovo exposures of chick embryos to iAs and phenytoin also induced lethality in 24% of the embryos (Table 1).

To specifically test the in silico prediction that the GR signaling pathway mediates the occurrence of structural birth defects caused by iAs, the GR pathway was blocked using the inhibitor cortexolone. Embryos exposed to cortexolone alone or to phenytoin plus cortexolone revealed the same malformations as embryos exposed to phenytoin alone. The combination of phenytoin plus cortexolone also induced lethality in 24% of the embryos (Table 1).
cortisolone had no gross structural malformation and developed normally (Figure 2D,E, Table 1). Surprisingly, embryos exposed to iAs plus cortisolone showed no gross structural malformations and displayed normal growth parameters (Figure 2F, Table 1).

Embryo growth (crown–rump length) was significantly \( p < 0.05 \) decreased in phentoin-exposed and iAs-exposed embryos compared with controls, whereas cortisolone-treated embryos developed normally and were comparable to the control group (Figure 3A).

In addition, statistical analyses of other morphological scoring criteria showed a significant difference \( p < 0.05 \) between iAs or phentoin and the control groups for vitelline circulation, flexion of the embryo, brain development, and craniofacial development (Figure 3B–E).

No gross malformations were detected in heart or limbs for any treatment group (see Supplemental Material, Figure S3A,B [http://dx.doi.org/10.1289/ehp.1205659]).

Mediation of iAs-induced gene expression changes by GR. To validate that iAs influences gene expression via the GR pathway in the developing chick, we performed qRT-PCR. We assessed the expression levels of two transcription factors, \( NF-kB1 \) and \( AP1 \), in the head regions of the embryos. \( NF-kB1 \) (fold change = 3.85) and \( c-FOS \) (fold change = 5.66) were up-regulated in embryos treated with iAs (0.1 \( \mu M \)) [see Supplemental Material, Figure S4A,B (http://dx.doi.org/10.1289/ehp.1205659)].

Embryos treated with iAs plus cortisolone showed restored levels of \( NF-kB1 \) \( p < 0.01 \) and \( c-FOS \) \( p < 0.05 \) (see Supplemental Material, Figure S4A,B). In phentoin-treated embryos, gene expression levels of \( NF-kB1 \) and \( c-JUN \) were not significantly changed, but \( c-FOS \) expression was increased. Surprisingly, cortisolone did not block the effects of phentoin on the expression level of \( c-FOS \). These results show that other genes/proteins in the GR signaling pathway may influence phentoin and cortisolone and their interactions in this model system. The increased \( c-JUN \) expression level was not statistically significant in any of the treatment groups (see Supplemental Material, Figure S4C).

Mediation of iAs and Cd-induced neurodevelopmental toxicity by the GR pathway. The GR pathway was predicted to mediate the response to Cd, Hg, iAs, and Se. Thus, as a further test of the role of the GR in mediating metal-induced neurodevelopmental toxicity, we chose to test Cd as well as iAs. Embryonic chick midbrain MM cultures were exposed to iAs (1–5 \( \mu M \)), Cd (1–8 \( \mu M \)), phentoin (100 \( \mu M \)), or cortisolone (2 \( \mu M \)). We used 5-Fu and PenG, respectively, as positive and negative controls for cytotoxicity. The 8 \( \mu M \) concentration of 5-Fu resulted in a significant \( p < 0.01 \) reduction in cytotoxicity (Figure 3F). This suggests that differentiating midbrain MM cultures were susceptible to the toxic effects of 5-Fu at this concentration. As expected, PenG was not cytotoxic to the midbrain MM cultures. Exposure of the cultures to phentoin or phentoin plus cortisolone did not result in any cytotoxicity. This is consistent with the results of Regan et al. (1990), who observed no cytotoxic effects in primary neuronal cells treated with this concentration of phentoin. We observed a significant decrease in the viability of embryonic midbrain cells treated with 2 and 5 \( \mu M \) iAs or 5 and 8 \( \mu M \) Cd (Figure 3F).

Cortisolone-treated cells, with inhibited signaling of the GR pathway, were protected from cytotoxicity induced by 2 \( \mu M \) iAs. At the highest iAs concentration (5 \( \mu M \)), midbrain MM cultures were partially protected from iAs-induced cytotoxicity. Cortisolone completely protected the midbrain cells from the cytotoxic effects of Cd (Figure 3F).

Discussion

Congenital malformations are the leading cause of infant mortality. An estimated 120,000 infants are born with severe congenital malformations each year in the United States (Brent 2004). Of all the congenital malformations, 60–70% are caused by unknown environmental and/or genetic causes (Moore and Persaud 1998). Thus, there is a need for effective methods to identify biological pathways that are pathophysiologically related to birth defects. There is great promise that systems biology can be used to identify biological pathways that relate environmental contaminants with human development/diseases (Chuang et al. 2010) and birth defects (Sperling 2011).

In the present study, we developed an in silico strategy to aid in the understanding of metal-induced birth defects. To investigate the relationship between metal exposures and birth defects, we applied this novel approach whereby genes associated with seven selected metals were identified using the CTD. To identify genes with any known relationship to birth defects, the CTD-retrieved gene/protein data sets were then filtered for only those genes with known associations to embryonic development or developmental disorders. Biological pathways associated with developmental disorders were further elucidated using a systems-level analysis.

Although other studies have used the CTD to identify metal-perturbed pathways (Davis et al. 2008; Mattingly et al. 2009), we introduced a health outcome filter to enrich for relationships to structural birth defects that may result from exposure to toxic metals. The metal- and development-associated genes were highly enriched for 22 canonical pathways, many of which were common to multiple metals. For example, the GR signaling pathway was identified as a top ranking pathway for Cd, Hg, iAs, and Se. In addition, the GR pathway was also generally enriched in the genes/proteins identified for Cr, Ni, and Pb.

Figure 2. Photographs of representative embryos from each treatment group showing morphological features of chick embryos treated in ovo. Developmental end points were assessed between treatment and control groups on day 8. (A) Control (PBS-treated) embryo showing the development of forebrain (FB), midbrain (MB), hindbrain (HB), eye (E), heart (H), fore limb buds (FL), and hind limb buds (HLL). (B) Phentoin (PHT)-treated embryo exhibiting an abnormal head shape, failure of closure of the anterior part of the neural tube (+), and craniofacial defects (*). (C) iAs\footnote{125} treated embryo exhibiting craniofacial and anterior neural tube defects (anencephaly) (*). Embryos treated with (D) cortisolone (CX), (E) PHT plus CX, or (F) iAs\footnote{133} plus CX (A). Bar = 2 mm.
This finding is supported by other reports that metals, including Cd, Cr, Hg, iAs, Pb, and Se, influence signaling of the GR signaling pathway (Webster Marketon and Sternberg 2010). Although these metals have been shown to be associated with the GR, the role of this pathway in mediating metal-induced developmental defects is understudied. In addition to the GR pathway, other pathways, such as that mediated by AhR, were significantly enriched for Cr and for phenytoin. These findings suggest other biological pathways that warrant future follow-up. These results may also provide a means by which biological pathways that mediate the developmental effects of metals are prioritized for study.

In general, structural birth defects are believed to result from complex mechanisms, including multiple genes and signaling pathways (Mitchell 2005). The GR pathway exemplifies this complexity and is composed of pro-inflammatory cytokines, enzymes, cell adhesion molecules, and transcription factors such as NF-κB1 and AP-1. Our results show that many of the genes/proteins involved in the GR pathway are enriched for interactions of two or more of the metals. For example, NF-κB1 is associated with Cd, Hg, iAs, Ni, Pb, and Se (CTD 2011).

We postulated that the GR signaling pathway may be a biological pathway that associates metals with resultant structural birth defects. To test this computational prediction, we prioritized one of the selected study metals for testing using the whole chick embryo culture in ovo model. We selected iAs for further investigation because it is a well-established developmental toxicant and is listed as the highest priority hazardous substance by the ATSDR (2010). iAs can readily cross the human placenta and accumulate in fetal neuroepithelium junction, thereby plausibly inducing various developmental defects (Wlodarczyk et al. 1996, 2006).

In the present study, we observed that low levels of iAs (0.1 µM, or 7.5 ppb) induced structural birth defects including microcephaly, anterior neural tube defects (anecephaly), and gross craniofacial defects in the chick embryo. These results are similar to those from previous studies that showed iAs-induced structural malformations in the chick (Han et al. 2011) and mouse embryo models (Chaineau et al. 1990; Tabocova et al. 1996). The iAs-induced malformations in mouse have also been shown to be associated with systems-level changes in gene expression (Robinson et al. 2011).

Although strong evidence demonstrates the teratogenic effects of iAs in animal models, debate remains as to the effects on human congenital malformation (DeSesso 2001; Golub et al. 1998; Holson et al. 2000). Potential epidemiologic associations relating iAs with birth defects have been reported in human populations (Kwok et al. 2006; Zierler et al. 1988). For example, iAs-associated low birth weight has been previously demonstrated in human populations (Hopenhayn et al. 2003). The iAs concentration used in the present study is within the 10 ppb (0.13 µM) maximum allowable level of arsenic in U.S. public drinking water supplies (U.S. Environmental Protection Agency 2012).

The mechanism by which iAs induces structural birth defects in animal models is largely unknown. Given the results of our computational analyses, we postulated that iAs may induce birth defects via the GR signaling pathway. To test this, we used the known GR inhibitor cortexolone. Cortexolone binds to the GR and leads to an altered conformational

**Figure 3.** Embryonic growth, morphological scores, and toxicity of chemical compounds in whole chick embryos cultured in ovo (incubation day 3–6). Crown–rump length (A), vitelline circulation (B), flexion (C), brain (D), and gross facial deformities (E) of embryos treated with PBS (control), vehicle control (0.1% ethanol), phenytoin (PHT), iAs³⁻, cortexolone (CX), PHT plus CX, or iAs³⁻ plus CX. (A) Effects of iAs³⁻ and Cd (CdCl₂) on cell viability of midbrain MM cultures 5 days after exposure; positive and negative controls for cytotoxicity were 5-Fu and PenG, respectively. Data represent the means ± SEs from four independent experiments (n = 4).

*p < 0.05, **p < 0.01, and ***p < 0.001 compared with control and vehicle groups. #p < 0.05 compared with 2 and 5 µM iAs³⁻ alone or 5 and 8 µM Cd alone.
form of the GR complex, which is then transported into the nucleus (Kaiser et al. 1972). We observed that blockade of the GR pathway indeed prevents the iAs-induced craniofacial and neural tube defects. Others have reported that corticosterone successfully reduces the occurrence of phenytoin-induced birth defects in mouse embryos (Kay et al. 1990). Corticosterone also prevents GR-mediated teratogenesis (e.g., cleft palate, limb defects) in chick embryos (Jelinek et al. 1983; Pavlik et al. 1986). To our knowledge, no previous studies have examined GR blockade as a means for prevention of metal-induced birth defects.

Our results show a relationship among iAs, birth defects, and the GR pathway. iAs has a biphasic effect on GR function and disrupts GR-mediated transcription in a complex fashion. Specifically, very low doses of iAs (e.g., 0.01 μM, or 0.7 ppb) have been shown to alter the function of the GR as a transcription factor, enhancing GC induction of endogenous GR-regulated genes (Bodwell et al. 2004; Davey et al. 2007; Kaltreider et al. 2001). Studies have also shown that low doses of iAs (e.g., 0.1 μM, or 7.5 ppb) can interfere with hormone receptor binding and can act as a potent endocrine disruptor of hormone-mediated gene transcription by the GR (Bodwell et al. 2004, 2006; Davey et al. 2007; Kaltreider et al. 2001). Furthermore, iAs causes altered signaling via oxidative stress induction, which can damage DNA in cells by turning on heat shock protein production (Hughes 2002). Through these mechanisms, iAs may activate GR-mediated gene transcription. Several studies have demonstrated that iAs enhances NF-κB and AP1 DNA binding and induces stress responsive transcription factors that may play important roles in iAs-induced signal transduction, cell transformation, and apoptosis (Dong 2002; Drobna et al. 2003; reviewed by Zeng et al. 2005). In the present study, we examined the expression levels of GR-mediated NF-κB1 and AP1 and found that iAs increased their expression levels in the head region of the deformed chick. Their expression levels were muted in the presence of corticosterone, indicating that iAs influences the expression of these genes via the GR pathway. It is important to note that phenytoin, the GR-specific control, failed to impact the expression of NF-κB1, with only c-FOS showing a statistically significant change. These data suggest complex signaling responses to phenytoin that may act on other genes within the GR pathway.

GC-associated effects are mediated through the GR. Several possible mechanisms can cause the disruption of the GR signaling pathway, such as excessive amounts of GCs leading to intrauterine growth retardation and low birth weight (Drake et al. 2007). In the fetus, unmetabolized GCs appear to function as the active teratogenic agent (Goldman et al. 1978; Pratt 1985). GCs can also influence glycolysis via a GR-mediated mechanism (Loiseau et al. 1985). Evidence suggests that GC teratogenesis is a result of direct action on the embryo, which triggers a characteristic pattern of dysmorphogenesis via the biochemical and GC-mediated anti-inflammatory pathway (Kay et al. 1990; Pratt 1985).

The in silico results presented here indicate that not only iAs but other metals, such as Cd, may also act through the GR. To examine the links between the GR pathway and these two separate metals, we further assessed the toxicity of iAs or Cd in midbrain MM culture. An established method to assess the effects of developmental toxicants (L’Huillier et al. 2002). The results demonstrate that inhibition of GR signaling partially protected against iAs-induced cytotoxicity at the highest dose, while complete protection was observed at the lower doses. GR blockade completely protected against Cd-induced cellular toxicity. These data support the prediction that metals other than iAs also act via the GR pathway. Taken together, the results from this study show that iAs-induced structural birth defects are dependent on signaling through the GR pathway (Figure 4). These findings highlight an in silico method useful for the selection of a targeted biological pathway to test for birth defects prevention. The results suggest a plausible pathophysiological mechanism by which iAs alters the GR pathway to ultimately cause birth defects.

Conclusions

We used a systems biology–based computational approach to determine that the GR pathway is a highly enriched pathway integrating a panel of metals (e.g., Cd, Hg, iAs, Se) with birth defect–associated genes. On the basis of the computational prediction, we used a GR inhibitor to demonstrate that iAs-induced structural malformations can be prevented in the chick embryo model. In addition, iAs- and Cd-induced neurodevelopmental toxicity were partially or completely

![Figure 4. Proposed model for iAs-induced birth defects via the GR pathway. iAs appears to induce birth defects in the chick embryo as a result of signaling through the GR (also known as NR3C1 (nuclear receptor subfamily 3, group C, member 1)) pathway. Gene members of the GR pathway are shown in red (associated with embryonic development) or green (associated with developmental disorders). In embryos with inhibited signaling of the GR pathway via corticosterone, the iAs-induced birth defects are prevented.](image-url)
protected via GR pathway inhibition assessed using the midbrain MM culture assay. Thus, these results illustrate the potential use of systems biology–based predictions in teratology and developmental toxicology research. We anticipate that this novel strategy can be employed to predict other biological pathways that mediate environmentally induced birth defects. Moreover, as applied to environmental metals, this type of cost-effective approach could be applied to a wide range of other environmental contaminants. These data provide novel information that may be useful in the prevention and treatment of metal-induced birth defects.

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