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Systems Biology and Birth Defects Prevention:

Blockade of the Glucocorticoid Receptor Prevents Arsenic-Induced Birth Defects

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Abbreviations:

AHR	aryl hydrocarbon receptor
AP1	activating protein 1
ATSDR	agency for toxic substances and disease registry
c-FOS	FBJ murine osteosarcoma viral oncogene homolog
c-JUN	jun proto-oncogene
CTD	comparative toxicogenomics database
CX	cortexolone
GR	glucocorticoid receptor
iAs	inorganic arsenic
IPA	ingenuity pathway analysis
JNK	c-JUN N-terminal kinase
MM	micromass
NF- κ B1	nuclear factor kappa B1
PHT	phenytoin
ppb	parts per billion

ABSTRACT

Background: The biological mechanisms by which environmental metals are associated with birth defects are largely unknown. Systems biology-based approaches may help to identify key pathways that mediate metal-induced birth defects as well as potential targets for prevention.

Objectives: The objective of this study was first to apply a novel computational approach to identify a prioritized biological pathway that associates metals with birth defects. Second, our aim was to test in the laboratory setting whether inhibition of the identified pathway prevents developmental defects.

Methods: Seven environmental metals were selected for inclusion in the computational analysis, namely arsenic, cadmium, chromium, lead, mercury, nickel and selenium. An *in silico* strategy was used to predict genes and pathways associated with both metal exposure and developmental defects. The most significant pathway was identified and tested using an *in ovo* whole chick embryo culture assay. The role of the pathway as a mediator of metal-induced toxicity was further evaluated using the *in vitro* midbrain micromass (MM) culture assay.

Results: The glucocorticoid receptor pathway was computationally predicted to be a key mediator of multiple metal-induced birth defects. Using the chick embryo model, structural malformations induced by inorganic arsenic (iAs) were prevented by inhibiting signaling of the glucocorticoid receptor pathway. Further, glucocorticoid receptor inhibition demonstrated partial to complete protection to both iAs and cadmium-induced neurodevelopmental toxicity *in vitro*.

Conclusions: These findings highlight a novel approach to computationally identify a targeted biological pathway to test for birth defects prevention.

INTRODUCTION

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 with such exposure occurring as a result of diet, drinking water, airborne particles, consumer products, and certain occupational environments (Tabacova 1986). There is evidence suggesting that many toxic metals indeed cross the placental barrier (Al-Saleh et al. 2011; Casey and Robinson 1978; Concha et al. 1998; Guo et al. 2010; Rudge et al. 2009), and thus are likely to have a detrimental impact on the developing fetus.

The focus of this study was to select seven high priority toxic metals and/or metalloids based on their presence in the environment and their known or suspected role as developmental toxicants. The following were selected for study: cadmium (Cd), chromium (Cr), inorganic arsenic (iAs), lead (Pb), mercury (Hg), nickel (Ni) and selenium (Se). Four of these, namely Cd, Hg, iAs and Pb are ranked in the top ten most hazardous substances by the Agency for Toxic Substances and Disease Registry (ATSDR) (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; Messerle and Webster 1982; Tabocova 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 in drinking water has been associated with adverse pregnancy outcomes such as spontaneous abortion, still birth and preterm birth (Ahmad et al. 2001), as well as congenital malformations (Kwok et al. 2006; Zierler et al. 1988).

Clearly, there is public health concern surrounding environmental exposure-mediated birth defects supported by both epidemiological and animal-based evidence. Still, the underlying pathophysiological mechanisms linking prenatal exposures to developmental disorders remain largely unknown. In this study we set out to test the hypothesis that biological pathways that mediate metal-induced birth defects could be uncovered by identifying common signaling pathways that integrate both metal- and development-associated genes. The study applied a systems biology approach coupled with a teratogenic experimental strategy.

Specifically, the research framework included: (i) the identification of gene-contaminant relationships from a comparative toxicogenomics database; (ii) the prediction of biological pathways associated with metals exposure and developmental disorders, and (iii) laboratory-based validation of the *in silico* pathway prediction. This novel computational approach was applied to the seven metals of interest and resulted in the prediction that the glucocorticoid receptor signaling pathway may be a key mediator highly associated with four of the selected metals, specifically Cd, Hg, iAs and Se. Focusing on this pathway, the *in ovo* chick embryo culture model was used to demonstrate that structural malformations induced by one of the metals, iAs, can be prevented through blockade of the glucocorticoid receptor signaling pathway. In addition, an *in vitro* micromass (MM) culture assay was used to demonstrate that both iAs and Cd-induced neurodevelopmental toxicity were partially or completely prevented by blockade of the pathway. These results provide evidence for a novel systems biology strategy by which biological pathways can be predicted and subsequently tested to increase our understanding of pathophysiological mechanisms related to birth defects.

MATERIALS AND METHODS

Identifying metal-associated genes

To identify genes known to be associated with the metals of study the Comparative Toxicogenomics Database (CTD) was used (Davis et al. 2011; CTD 2011). The CTD is a manually curated toxicogenomic database, including over 178,000 interactions between 4,980 chemicals and 16,182 genes/proteins in 298 species. It contains 8,900 gene/protein-disease direct relationships, and 5,600 chemical-disease relationships (CTD 2011; Davis et al. 2011). The CTD Batch Query tool was used to retrieve all curated chemical-gene/protein interactions for each of the seven selected metals separately (e.g. Cd, Cr, Hg, iAs, Ni, Pb, and Se) (CTD 2011). In addition, the CTD was used to identify genes/proteins associated with phenytoin, a well-known human teratogen (Buehler et al. 1990) which served as a positive control for the *in ovo* experimentation.

Identifying metal-associated genes with roles in development

Once metal-associated genes/proteins were identified using the CTD database, biological function enrichment analysis was performed using the 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 (Redwood City, CA). 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. Metal-associated genes were mapped in IPA to their global molecular networks, where 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 the 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, to test the computational prediction that the glucocorticoid receptor signaling pathway is involved in metal-induced developmental disorders, *in ovo* whole chick embryo culture was used as it is a well-established model for teratogenicity assessment (Kucera et al. 1993). All experimental procedures were conducted on embryos less than ten days of age and thus were exempt from oversight by the UNC Institutional Animal Care and Use Committee. Fertilized white leghorn chicken eggs were obtained 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 prepared as follows: (1) control (PBS only), (2) vehicle control (0.1 % ethanol), (3) phenytoin, (Fisher Scientific, Waltham, MA, USA) as a positive control for neural tube defects, (4) iAs as sodium arsenite (iAs^{3+}) (Sigma-Aldrich, St. Louis, MO, USA), (5) cortexolone (Fisher Scientific, Waltham, MA, USA), (6) phenytoin plus cortexolone, and (7) iAs^{3+} plus cortexolone. Ten to twelve embryos

were examined per treatment group in four independent biological replicates. Eggs were incubated at 100^oF with relative humidity of 55%. The day on which the eggs were incubated was counted as day 0. On day 3 of incubation the eggs were dosed as follows: after being swabbed with 70% ethanol the blunt end of the eggs was stuck with forceps to make a small hole using an established protocol (Kucera et al. 1993; Memon and Pratten 2009). Control embryos were treated with the same volume of phosphate buffered saline (1X PBS) without any chemicals. Eggs received 100 μ l injections of PBS, ethanol (0.1%), phenytoin (400 μ M), iAs³⁺ (0.1 μ M or 7.5ppb), cortexolone (2 μ M), phenytoin plus cortexolone, or iAs³⁺ plus cortexolone, and were incubated up to day 6. The concentration of cortexolone was selected based upon previous literature (Harlow et al. 1987; Turnell et al. 1974). On day 6, embryos were removed and analyzed for gross malformations and scored according to established morphological scoring criteria (described by (Memon and Pratten 2009)) (Supplemental Material, Table S1). Only viable embryos, defined by the presence of a beating heart and visibly circulating blood in the embryo and yolk 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). A Kruskal-Wallis one-way ANOVA followed by Mann Whitney U-test for non-parametric data was used to assess the morphological scoring criteria (vitelline circulation, flexion, heart, brain, gross facial deformities and limbs) between exposure groups. The statistical process was carried out as in Hewitt et al. (2005). The mean and standard error of the morphological scores were determined for the four independent experiments. Embryo growth, a measurement of crown-rump length, was analyzed using parametric statistical tests (ANOVA followed by Dunnett's post hoc testing). For the statistical testing, *p*-value was set at 0.05.

Quantitative reverse-transcription polymerase chain reaction (q-RT-PCR)

The gene expression levels of two transcription factors, namely *NF-κB1* and *API* were assessed. *API* is comprised of *c-FOS* and *c-JUN* and these genes were assessed relative to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) using quantitative real-time PCR (q-RT-PCR) with primer sequences as shown in Supplemental Material, Table S2. Total RNA was isolated from the whole head regions of treated and untreated embryos (n=3 embryos/group) using an RNeasy[®] 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 control group were calculated based on delta-delta cycle threshold ($\Delta\Delta Ct$) values and normalized with *GAPDH* as a housekeeping gene. Statistical significance of the treatment group versus control group transcript levels was calculated using an unpaired *t*-test.

Chick embryo midbrain micromass culture in vitro assay

The role of the glucocorticoid receptor pathway as a mediator of metal-induced neurodevelopmental toxicity was further evaluated using the *in vitro* chick embryo midbrain micromass (MM) culture assay. The midbrain MM culture assay is a well-accepted method to screen for developmental toxicity of compounds and was prepared according to previously established protocols (Flint, 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, 200mM L-glutamine and 50units/ml Penicillin, 50μg/ml Streptomycin. Cell density was estimated using a hemocytometer and adjusted to

1×10^6 cells/ml. A total of 10 μ l of the cell suspension was pipetted onto the center of a collagen-coated well in a 96-well culture plate. After 24h, midbrain MM cultures were treated with or without test chemicals and appropriate controls: iAs^{3+} (1, 2, and 5 μ M), cadmium as $CdCl_2$ (1, 2, 5, and 8 μ M), phenytoin (100 μ M), cortexolone (2 μ M), a combination of cortexolone with iAs^{3+} or $CdCl_2$, or cortexolone plus phenytoin. The assay included positive and negative controls for cytotoxicity, namely 5-fluorouracil and penicillin G, respectively. Chick embryo midbrain MM culture cytotoxicity was measured after 5 days in culture using the one step resazurin reduction assay (O'Brien et al. 2000). The optical density was measured on a SynergyTM HT Multi-Mode Microplate Reader (BioTek, Winooski, VT) with an excitation filter 530 \pm 25nm, and emission filter 590 \pm 35nm giving an indication of cell viability/cell culture metabolic activity. Statistical analysis was performed using Graphpad Prism 5 software (San Diego, CA). For comparison between two groups, a Student's *t*-test was performed. For comparisons among groups, a one way ANOVA followed by an appropriate post hoc test (e.g. Dunnett or Bonferroni) was used. A *p*-value <0.05 was considered statistically significant.

RESULTS

A novel approach to identify biological pathways that may mediate environmentally-induced birth defects was employed. Specifically this strategy involved identifying genes associated with environmental metals using the CTD, filtering these genes for biological function related to birth defects/development, and examining the genes for known biological interactions in the cell. This computational approach is graphically represented (Supplemental Material, Figure S1).

Metal and development-associated genes identified

The *in silico* approach involved first the identification of metal-associated genes. A total of seven metals were selected for study, namely Cd, Cr, Hg, iAs, Ni, Pb and Se. Genes with known association to the metals of interest were obtained from the CTD (Supplemental Material, Table S3). The genes contained within the CTD represent broad relationships with environmental toxicants (here queried for metals) and include known effects on gene expression but also genes encoding proteins that have altered metal-associated changes in protein abundance and protein activity. Thus, for each of the metals a number of genes were identified with known relationship to Cd (n=518 genes), Cr (n=175 genes), Hg (n=334 genes), iAs (n=1880 genes), Ni (n=637 genes), Pb (n=774 genes), and Se (n=1616 genes) (Supplemental Material, Tables S3 and S4). In addition, genes associated with phenytoin, a known teratogen, were also identified as a control (n=138) (Supplemental Material, Tables S3 and S4).

The metal-associated gene lists were subsequently filtered for genes/proteins with known relationships broadly to organismal development/birth defects. Specifically, biological function enrichment analysis was performed, and genes related to either “embryonic development” or “developmental disorders” were identified and extracted from within the metal-associated gene lists. The resultant numbers of genes that were thus identified as both metal- and development-associated ranged in count from 76 to 604 (Supplemental Material, Tables S3 and S5).

Pathways influencing metal-induced developmental disorders were predicted

Biological pathways that were enriched amongst the metal- and development-associated genes were algorithmically constructed. For each of the metals and for phenytoin the highest

ranking (e.g. top five) canonical pathways were selected. As there was some overlap, this resulted in a total of 22 unique canonical pathways. These pathways are displayed (Figure 1) and a complete list of all top ranking pathways with their p -values is presented (Supplemental Table S6). The pathway analysis revealed that, in general, distinct pathways are highly enriched among genes associated with each of the metals (Figure 1). Still, many of the pathways were enriched between two or more of the metals. The glucocorticoid receptor (GR) signaling pathway was identified as high ranking within the top canonical pathways for Cd, Hg, iAs and Se. Notably, this indicates that genes associated with these four metals- 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 related to the metals- 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, a subsequent analysis was performed 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 (Supplemental Material, Figure S2). 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, nuclear factor kappa B1 (NF- κ B1), a protein involved in the GR signaling pathway, has a known relationship to six of the seven metals (CTD 2011). Likewise, activating protein 1 (AP1) (comprised of c-FOS, c-JUN) and c-JUN N-terminal kinase (JNK) are also known to be associated with multiple metals (CTD 2011) (Supplemental Material, Figure S2).

Embryo culture assay validates computational prediction

Given the computational analysis predicting association of the metals (e.g. 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. The hypothesis was that the GR mediates metal-induced defects. One of the metals, iAs, was prioritized for testing and results were compared to the known teratogen phenytoin, a GR-dependent teratogen (Kay et al. 1990). A total of ten to twelve 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% of the embryos (Table 1). No phenotypic abnormalities were observed in embryos treated with PBS or the vehicle control (Figure 2A, Table 1). For phenytoin and iAs, morphological defects were observed including abnormalities in the head fold region, microcephaly, anterior neural tube defects and gross facial deformities (Figure 2B, 2C). Specifically, the craniofacial defects included altered optic and beak 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 phenytoin combined with cortexolone did not show any gross structural malformation and developed normally (Figure 2D, 2E, Table 1). Strikingly, embryos exposed to iAs but where the GR signaling pathway was

inhibited via cortexolone 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 phenytoin-exposed and iAs-exposed embryos compared to controls, while similarly exposed embryos treated with cortexolone developed normally and were comparable to the control group (Figure 3A). Additionally, statistical analyses of other morphological scoring criteria showed a significant difference between iAs or phenytoin and the control treatment groups for vitelline circulation, flexion of the embryo, brain development, and craniofacial development (Figure 3B-3E) ($p < 0.05$). There were no gross malformations detected for the heart or limbs for any of the treatment groups (Supplemental Material, Figure S3A and B).

GR mediates iAs-induced gene expression changes

To validate that iAs influences gene expression via the GR pathway in the developing chick, q-RT-PCR was performed. Using the head regions of the embryos, the expression levels of two transcription factors *NF- κ B1* and *API* (comprised of *c-FOS* and *c-JUN*) were assessed. *NF- κ B1* (fold change = 3.85) and *c-FOS* (fold change = 5.66) were up-regulated in embryos exposed to iAs (0.1 μ M) (Supplemental Material, Figure S4A and S4B). Embryos exposed to iAs but with inhibited signaling of the GR pathway via cortexolone showed restored levels of *NF- κ B1* ($p < 0.01$) and *c-FOS* ($p < 0.05$) (Supplemental Material, Figure S4A and S4B). Phenytoin treatment did not result in a significant change in gene expression levels of *NF- κ B1* nor *c-JUN* but did result in increased expression of *c-FOS*. Surprisingly, cortexolone did not block the effects of phenytoin on the expression level of *c-FOS*. These results suggest that other

genes/proteins in the GR signaling pathway may influence phenytoin and cortexolone and their interactions in this model system. The increased expression level of *c-JUN* was not statistically significant in any of the treatment conditions (Supplemental Material, Figure S4C).

GR pathway mediates iAs and Cd-induced neurodevelopmental toxicity

The GR pathway was predicted to mediate the response to the following metals Cd, Hg, iAs, and Se. Thus, as a further test of the role of the GR in mediating metal-induced neurodevelopmental toxicity, a second metal was selected for testing. Embryonic chick midbrain micromass (MM) cultures were exposed to iAs (1-5 μ M), Cd (1-8 μ M), phenytoin (100 μ M) or cortexolone. In addition, 5-flurouracil (5-Fu) and penicillin G (PenG) were used as a positive and negative control for cytotoxicity, respectively. Concentrations of 8 μ M 5-Fu resulted in a significant ($p<0.01$) reduction in cytotoxicity (Figure 3F). This suggest that differentiating midbrain MM cultures were susceptible to the toxic effects of 5-Fu at the 8 μ M concentration. As expected, PenG was not cytotoxic to the midbrain MM cultures. Exposure of midbrain MM cultures to phenytoin or phenytoin plus cortexolone did not result in any cytotoxicity. This is consistent with Regan et al. (1990) which also showed that phenytoin at this concentration did not cause any cytotoxic effects in primary neuronal cells. There was a significant decrease in the viability of the iAs or Cd-treated cells (Figure 3F). Cells with inhibited signaling of the GR pathway via cortexolone were protected from iAs-induced cytotoxicity at the low concentration (2 μ M). At the higher concentration (5 μ M) of iAs the embryonic midbrain MM culture cytotoxicity was partially protected from iAs-induced cytotoxicity. Cortexolone 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% of birth defects are caused by unknown environmental and/or genetic causes (Moore and Persaud 1998). Thus, there is a current 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 this research, we developed an *in silico* strategy to aid in the understanding of metal-induced birth defects. In order 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 datasets were then filtered for only those with known associations to embryonic development or developmental disorders. Biological pathways associated with developmental disorders were further elucidated using a systems level analysis.

While other studies have used the CTD to identify metal-perturbed pathways (Davis et al. 2008; Mattingly et al. 2009), here we have 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. This finding is supported by other reports that metals including Cd, Cr, Hg, iAs, Pb and Se are known to influence signaling of the GR signaling pathway (Webster Marketon and Sternberg 2010). While these metals were previously 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, it is notable that other pathways such as that mediated by AHR were significantly enriched for Cr as well as 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 defects 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 comprised of pro-inflammatory cytokines, enzymes, cell adhesion molecules and transcription factors such as NF- κ B1 and AP1. Our results demonstrate 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- κ B1 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. iAs was selected for further investigation as it is a well-established developmental toxicant and is listed as the highest priority hazardous substance by the ATSDR (ATSDR 2010). It is known that iAs can readily cross the human placenta and accumulates in fetal neuroepithelium junction, thereby plausibly inducing various developmental defects (Włodarczyk et al. 2006; Włodarczyk et al. 1996).

Here, low levels of iAs (0.1 μ M or 7.5 ppb) were shown to induce structural birth defects including microcephaly, anterior neural tube defects (anencephaly), and gross craniofacial defects in the chick embryo. These results are similar to those from previous studies that have shown 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).

It should be mentioned that while 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). To note, the concentration used in this study is within the 10 ppb (0.13 μ M) maximum allowable level of arsenic in U.S. public drinking water supplies (EPA 2012).

The mechanism by which iAs induces structural birth defects in animal models is largely unknown. Given the results of our computational analyses, it was postulated that iAs may induce birth defects via the GR signaling pathway. To test this, the known GR inhibitor cortexolone was used. Cortexolone binds to the GR and leads to an altered conformational form of the GR complex which is then transported into the nucleus (Kaiser et al. 1972). We demonstrate that blockade of the GR pathway indeed prevents the iAs-induced craniofacial and neural tube defects. Others have reported that cortexolone successfully reduces the occurrence of phenytoin-induced birth defects in mouse embryos (Kay et al. 1990). It also prevents GR-mediated

teratogenicity (e.g. cleft palate and 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 between iAs, birth defects and the GR pathway. It has been shown that 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 μ M or 0.7 ppb) have been shown to alter the function of the GR as a transcription factor, enhancing glucocorticoid 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 μ M 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; Davey et al. 2007; Kaltreider et al. 2001; Bodwell et al. 2006). 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. There are several studies that demonstrate that iAs enhances NF- κ B1 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 in Zeng et al. 2005). Here we examined the expression levels of GR-mediated *NF- κ B1* and *API* 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 cortexolone indicating that indeed 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- κ B1* with only

c-FOS showing statistically significant change. These data suggest complex signaling responses to phenytoin that may act on other genes within the GR pathway.

It is known that glucocorticoid (GC)-associated effects are mediated through the GR. There are several possible mechanisms that 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, un-metabolized 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 teratogenicity is due to 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, the toxicity was further assessed in midbrain MM cultures in response to exposure to either iAs or Cd. The MM culture assay is 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 higher concentrations and offered complete protection against Cd-induced cellular toxicity. These data support the prediction that other metals in addition to iAs 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

In summary, a systems biology-based computational approach was used to identify that the glucocorticoid receptor pathway is a highly enriched pathway integrating a panel of metals (e.g. Cd, Hg, iAs and Se) with birth defect-associated genes. Based on the computational prediction, a glucocorticoid receptor (GR) inhibitor was used 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 protected via GR pathway inhibition assessed using the *in vitro* midbrain MM culture assay. Thus, these results illustrate the potential use of systems biology-based predictions in teratology/developmental toxicology research. It is anticipated that this novel strategy can be employed to predict other biological pathways that mediate environmentally-induced birth defects. Moreover, here 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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Table 1: Frequency of abnormal embryos.

Group	Total No. Embryo Explant	Total No. Dead Embryos	Total No. Survival Embryos	Normal No. (%)	Abnormal No. (%)
Control	42	1	41	41 (100)	
Vehicle Control	42	3	39	39 (100)	
Phenytoin	42	10	32	14 (44)	18 (56)
Arsenic (iAs³⁺)	42	10	32	13 (41)	19 (59)
Cortexolone	42	2	40	40 (100)	
Phenytoin plus cortexolone	42	8	34	34 (100)	
Arsenic (iAs³⁺) plus cortexolone	42	5	37	37 (100)	

FIGURE LEGENDS

Figure 1: Canonical pathways associated with metal exposure and developmental disorders. A total of 22 canonical pathways were found to be highly enriched in genes/proteins contained in lists retrieved from the CTD for at least one of the selected metals or phenytoin (PHT) the positive control. The selected metals were arsenic (iAs), cadmium chloride (Cd), chromium (Cr), lead (Pb), mercury (Hg), nickel (Ni), and selenium (Se). Colors denote pathways that were present (red) or absent (blue) as high ranking (e.g. within the top most five canonical pathways) for each of the metals or phenytoin.

Figure 2: Morphological features of chick embryos treated *in ovo*. Scale bar: 1mm. Comparison of developmental endpoints assessed between exposure groups and control groups at day 6. Representative embryos are shown for: (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 (HL). (B) Phenytoin (PHT)-exposed embryo, exhibiting an abnormal head shape with failure of closure of the anterior part of the neural tube (+) and craniofacial defects (*). (C) Inorganic arsenic (iAs³⁺)-exposed embryo exhibiting craniofacial (*) and anterior neural tube defects (anencephaly) (#). (D) Cortisolone (CX)-exposed embryo. (E) PHT plus CX-exposed embryo or (F) iAs³⁺ plus CX.

Figure 3: Morphological scores, embryonic growth and toxicity. Chemical compound effects on embryonic development in whole chick embryo cultured *in ovo* (incubation day 3-6). (A)

Crown-Rump length. (B) Vitelline circulation. (C) Flexion. (D) Brain. (E) Gross facial deformities. (F) The effects of arsenic (iAs^{3+}) and cadmium ($CdCl_2$) on midbrain micromass culture cell viability, 5-days post exposure. Positive and negative controls for cytotoxicity included 5-fluorouracil (5-Fu) and penicillin G (PenG), respectively. Data represent the averages from four independent experiments (mean \pm S.E., n=4). Significance is indicated as $*p<0.05$, $**p<0.01$, $***p<0.001$ for comparisons of the control and vehicle groups.

Figure 4: Proposed model for iAs-induced birth defects via the glucocorticoid receptor pathway. The results of this study suggest that iAs induces birth defects in the chick embryo as a result of signaling through the glucocorticoid (GC) receptor (GR or NR3C1) pathway. Gene members of the GR pathway are shown in red (embryonic development-associated) or green (developmental disorder-associated). In embryos with inhibited signaling of the GR pathway via cortisone the iAs-induced birth defects are prevented. Abbreviation: NR3C1-nuclear receptor subfamily 3, group C, member 1 (Glucocorticoid Receptor).

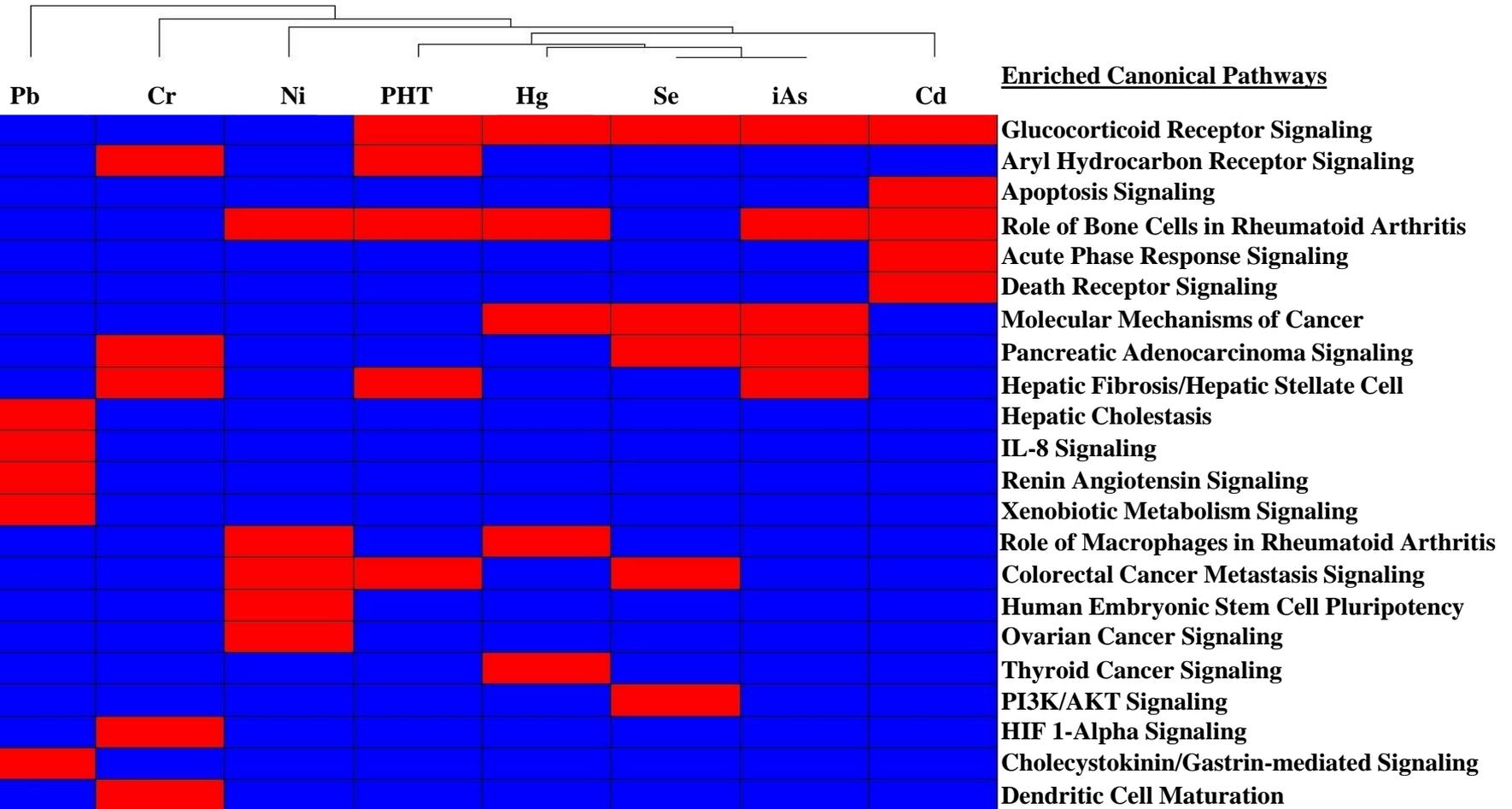


Figure 1

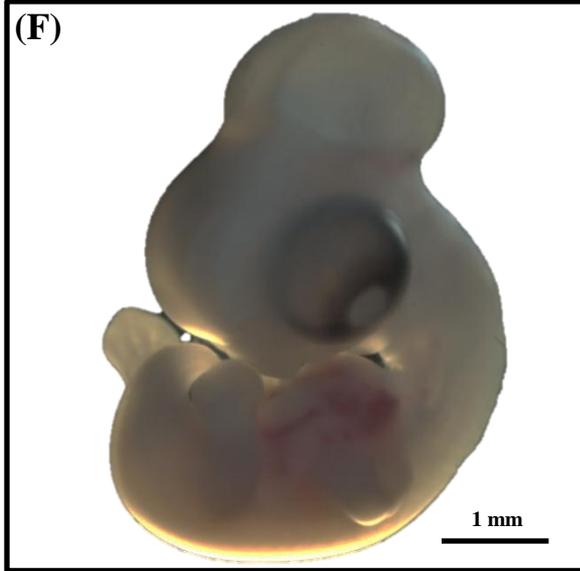
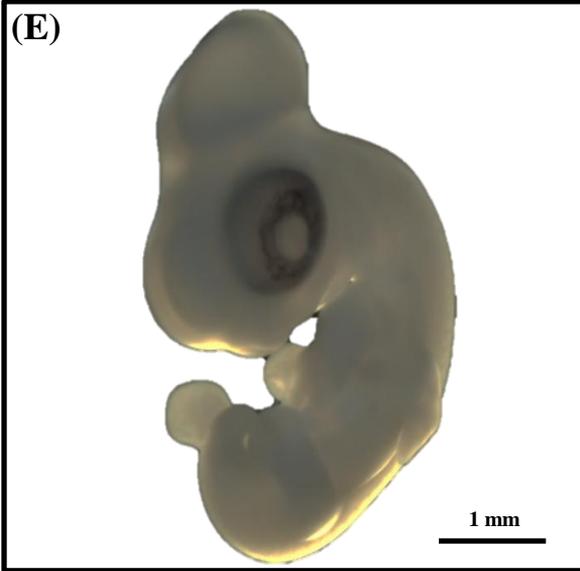
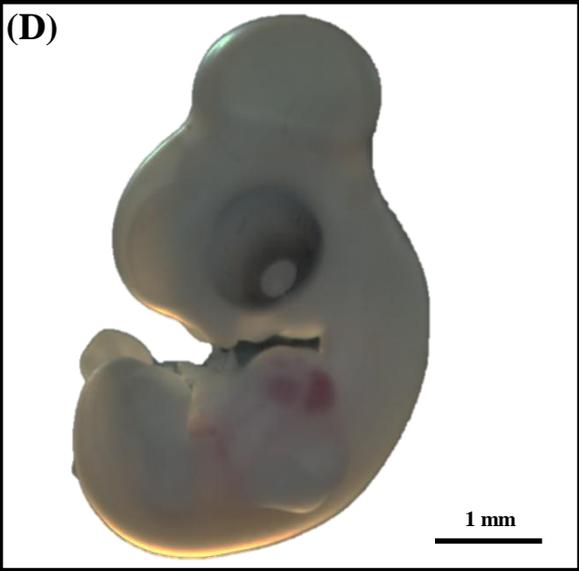
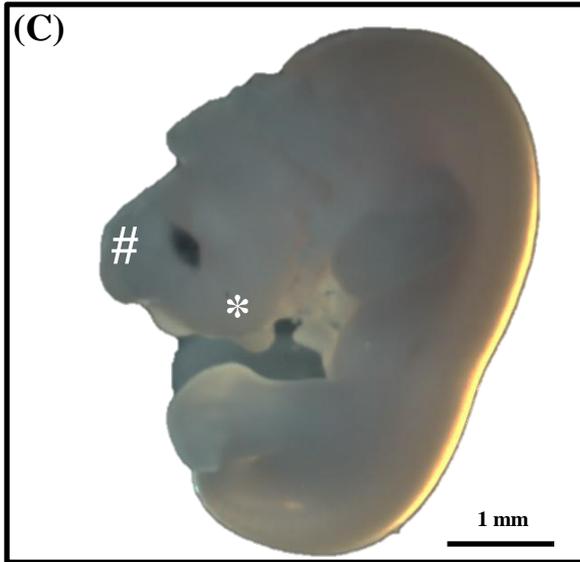
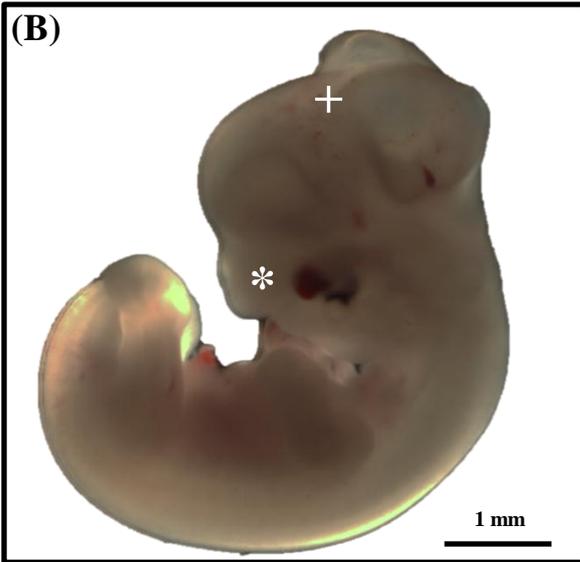
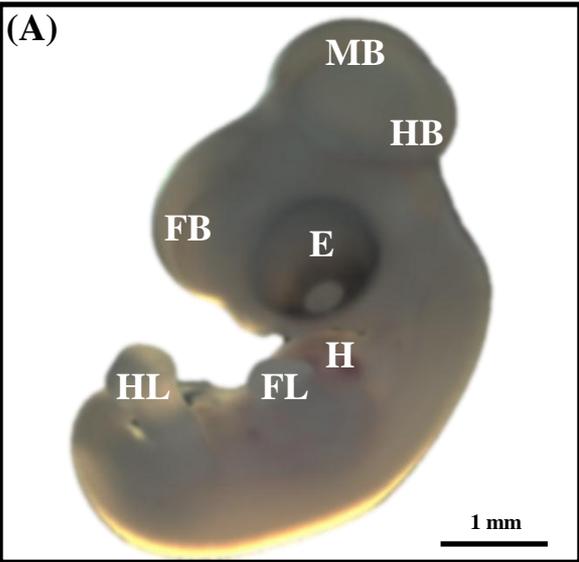


Figure 2

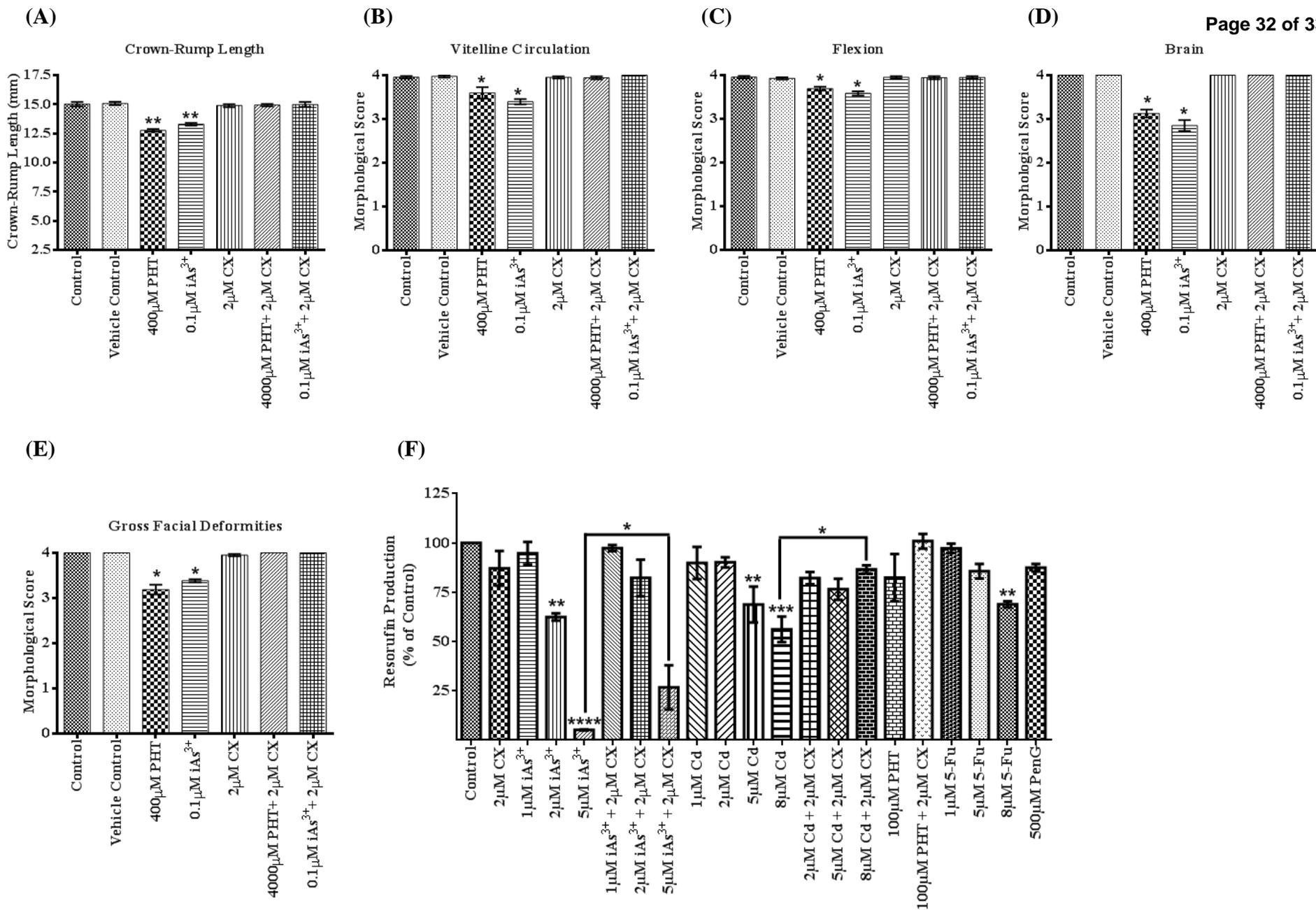


Figure 3

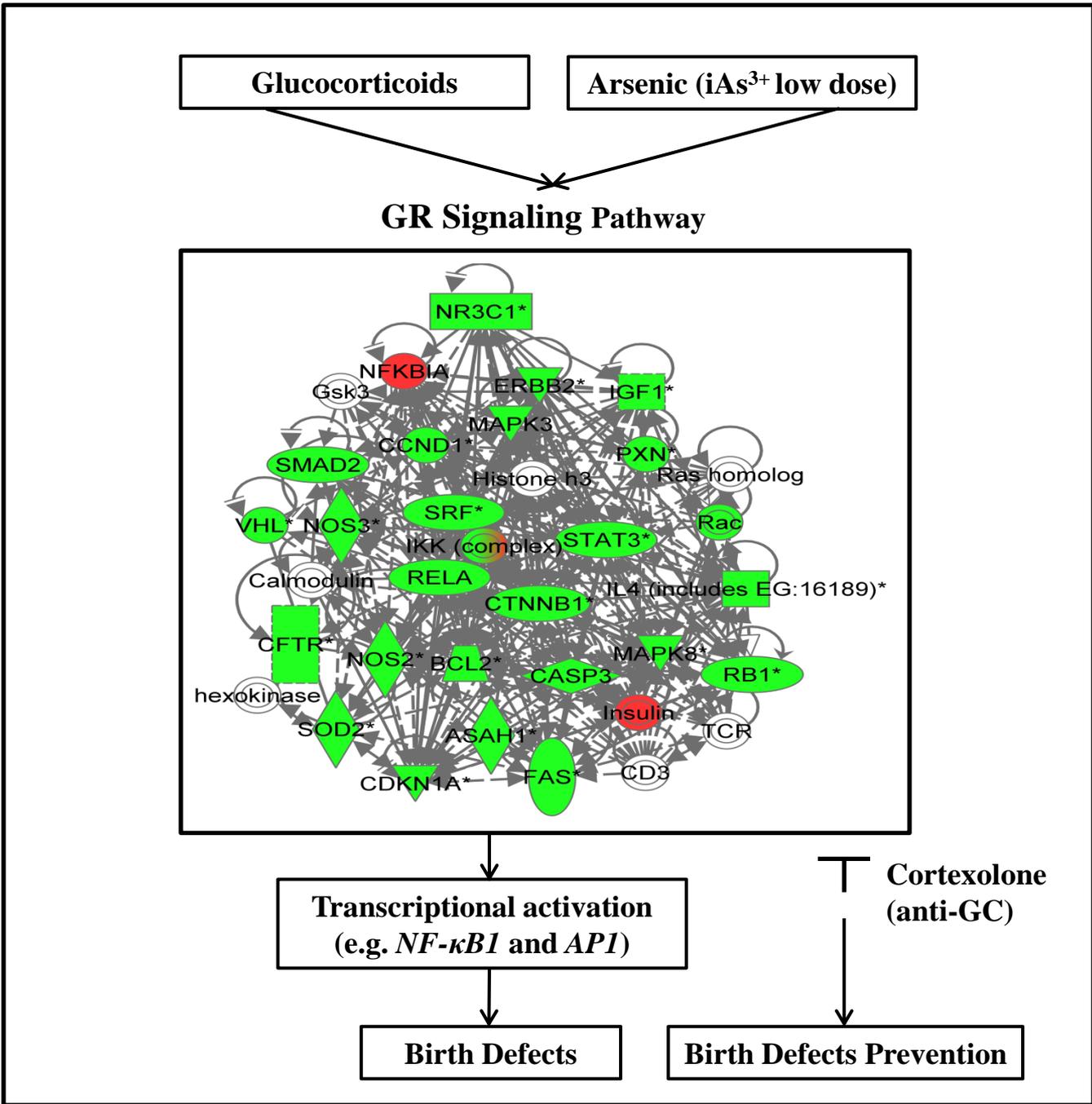


Figure 4