HOW CAN WE REDUCE THE EFFECTS OF ENVIRONMENTAL POLLUTANTS ON THE PLACENTA?

by

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Prenatal exposure to endocrine disrupting chemicals can lead to birth defects and adult disease. While it is impossible to avoid pollutant exposure, development of a prenatal supplement that augments natural protective mechanisms would be invaluable. Here, I show that prenatal supplementation with sulforaphane reduces gene changes caused by a model endocrine disrupting chemical. Further the candidate mechanisms of resilience associated with this rescue were identified.

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Nrf2: Potential for Enhanced Protection Against Environmental Pollutants

All living organisms are equipped with mechanisms to protect themselves from environmental insults. Nevertheless, environmental pollutants have become a major health concern. Pollutant exposure has direct negative effects on health but also is known to induce birth defects and induce developmental changes that lead to adult disease (Alias, et al., 2019) (Fernandez, et al., 2007) (Hanson & Gluckman, 2008). Although we have not evolved to detoxify each pollutant we are now exposed to, we do have cellular mechanisms that buffer the effects of environmental pollutants generally. One such protective mechanism is the Nrf2 signaling pathway, which has evolved over millions of years to protect against natural toxins and oxidative stress damage and contributes to cellular and tissue homeostasis (Telkoparan-Akillilar, Suzen, & Saso, 2019). In this review, I provide evidence that shows increased Nrf2 activation can be used as a mechanism of resilience against environmental pollutants.

The Nrf2 signaling pathway has several characteristics that make it an ideal candidate for a mechanism against environmental pollutants. The human Nrf2 gene was first identified and characterized in 1994, and encodes for the NRF2 protein which consists of 605 amino acids (Telkoparan-Akillilar, Suzen, & Saso, 2019). NRF2 is a transcription factor belonging to the leucine zipper factor family (CNC-bzip). Seven of Nrf2's functional domains, Neh1 through Neh7, are highly conserved. Neh1 is a region conserved in the CNC-bzip family, and binds to antioxidant response elements (AREs) (Telkoparan-Akillilar, Suzen, & Saso, 2019). AREs are found in the promoter regions of detoxifying and antioxidant enzyme genes and are responsible for the expression of phase II detoxifying enzymes (Kang, Lee, & Kim, 2005). Phase IIdetoxification enzymes catalyze reactions that break down dangerous metabolites and make

them easier to excrete. NRF2 targets AREs, making it an ideal target for upregulating detoxification and antioxidant pathways.

Several cellular stressors can induce NRF2 activation, promoting ARE-mediated transcription of detoxifying enzymes. These stressors include glutathione synthase deficiency, endogenous reactive oxygen species (ROS), and pro-oxidants (Kang, Lee, & Kim, 2005). In response to these stressors, Nrf2 translocates to the nucleus and associates with ARE to promote transcription of Phase II detoxifying enzymes. Phase II detoxifying enzymes are generally responsible for metabolic detoxification. This family of enzymes includes glutathione-S-transferase (GST), microsomal epoxide hydrolase, and VDP-glucuronyl transferase (Kang, Lee, & Kim, 2005). Phase II enzymes catalyze reactions that eliminate highly reactive carcinogens or radical intermediates, protecting cells from oxidative stress and preventing tissue damage (Simon, Haj-Yehia, & Levi-Schaffer, 2000) (Kang, Lee, & Kim, 2005). Thus, the Nrf2-ARE pathway promotes resiliency by buffering against cellular damage induced by oxidative stress.

Being able to reduce oxidative stress is important to prevent cellular toxicity and related health issues. Oxidative stress is defined as the imbalance between oxidants and antioxidants, that favors oxidants (Sies, 2000). Reactive Oxygen Species (ROS) are produced normally by endogenous systems, including enzymes within the electron transport chain (Braconi, Bernardini, & Santucci, 2011). At low levels ROS are involved in cellular signaling, but at high levels they become destructive to both DNA and proteins and cause cell and tissue damage (Simon, Haj-Yehia, & Levi-Schaffer, 2000). Extreme ROS is also known to induce cellular toxicity and apoptosis (Braconi, Bernardini, & Santucci, 2011) (Simon, Haj-Yehia, & Levi-Schaffer, 2000). Oxidative stress has been implicated in many health issues including preterm birth,

cardiovascular disease, and diabetes (Lakshmi, Padmaja, Kuppusamy, & Kutala, 2009) (Menon, 2014), therefore reducing its production is key to protection from disease.

ROS production is often enhanced by conditions of stress, including exposure to pollutants (Braconi, Bernardini, & Santucci, 2011). Heavy metals, air pollution (including diesel exhaust and cigarette smoke), pesticides, carbon monoxide, and asbestos, are all known to induce increased ROS (Franco, Sánchez-Olea, Reyes-Reyes, & Panayiotidis, 2009) (Upadhyay, Panduri, Ghio, & Kamp, 2003). This increased ROS often leads to oxidative stress and can lead to various health problems.

Understanding the mechanisms through which we protect ourselves from environmental contaminants and associated oxidative damage is imperative as we live in an increasingly polluted environment. NRF2 activation can contribute to the expression of proteins and upregulate systems that protect cells, tissues, and organisms from damage (Xue, et al., 2008). To understand the role of NRF2 in these systems we need to understand how NRF2 is activated, what NRF2 does after activation, and how it is currently being tested as a therapeutic.

Mechanism of NRF2 Regulation and Activation

The activity of NRF2 is regulated by several different pathways. The main pathway through which NRF2 is regulated is INrf2, or Keap1. Keap1 is a cytosolic inhibitor that exists as a dimer and keeps NRF2 retained in the cytoplasm. If Nrf2 is not activated, Keap1 binds to the Cul3/Rbx complex to induce Nrf2 ubiquitination (Kaspar, Niture, & Jaiswal, 2009). Under normal conditions NRF2 is continuously degraded by the Keap1/Cul3/Rbx1 complex (Kaspar, Niture, & Jaiswal, 2009). Although Keap1 is the main regulator of NRF2, the transcription factor can be regulated by alternate pathways. For example, Glycogen Synthase Kinase 3 (GSK-3β) can

regulate NRF2 by inducing ubiquitination. GSK-3β phosphorylates serine amino acid residues in the Neh6 domain of the protein leading to degradation. (Rada, et al., 2011). Thus, when NRF2 is not being activated or not needed, it is constantly degraded and non-active.

The activation pathway of NRF2 allows the transcription factor to be transported and bound to DNA. If NRF2 is activated, it is released from Keap1 and allowed to translocate to the nucleus (Su, et al., 2018) where it heterodimerizes with small Maf family proteins and binds to the ARE region to induce transcription (Kang, Lee, & Kim, 2005). The translocation of NRF2 is regulated by P13-kinase (Kang, Lee, & Kim, 2005). There are many ways Nrf2 can become activated, enhancing activation of Nrf2 is critical to being able to use it in a protective manner.

NRF2 Activators

Sulforaphane is a potent activator of NRF2. Sulforaphane acts by disassociating the transcription factor from Keap1 and allowing it to translocate to the nucleus. Sulforaphane is produced by the metabolism of glucoraphanin by the enzyme myrosinase (Fahey, et al., 2015). Glucoraphanin is found in cruciferous vegetables, including broccoli, kale, cabbage, and cauliflower (Kim & Park, 2016). Sulforaphane is shown to have many health benefits in humans, such as antiaging, improved organ function, and cancer chemoprotection (Yang, Palliyaguru, & Kensler, 2016). Many of these improved health benefits are provided through the reduction of oxidative stress via the Nrf2 pathway (Kikuchi, et al., 2015) (Townsend & Johnson, 2016).

6-methylsulfinylhexyl isothiocyanate (6-HITC), a chemical isolated from Japanese horseradish or wasabi, is another potent activator of NRF2 (Morimitsu, et al., 2002). 6-HITC has been shown to induce phase II detoxification by activating NRF2 and ARE transcription. This leads to lowered cancer risk and protection from oxidative damage (Morimitsu, et al., 2002). 6-

HITC has been proposed as a more potent activator of NRF2 than sulforaphane because it is absorbed so rapidly into the bloodstream. Because 6-HITC is a potent activator of NRF2, and therefore ARE transcription, it has the capability to reduce oxidative stress.

Sulforaphane and 6-HITC have been proposed as supplements to induce protection from environmental contaminants (Morimitsu, et al., 2002) (Telkoparan-Akillilar, Suzen, & Saso, 2019). Because Nrf2 is a transcription factor that induces transcription of phase II detoxification and antioxidant genes, it has many complex downstream effects. Finding the activators of Nrf2 that produce the intended downstream effects is an important goal to aid development of Nrf2 activators for enhancing protection from environmental pollutants. Understanding the effects of Nrf2 activation is immensely important in designing treatments that involve Nrf2-dependent protective systems.

Effects of NRF2 Activation

NAD(P)H: quinine oxidoreductase 1 (NQO1) is a gene that codes for a phase II detoxification product and is transcribed in result of NRF2 activation (Kim & Park, 2016). The product transcribed by NQQ1 is homodimeric flavoprotein (Zai, et al., 2010). Homodiemeric flavoprotein decreases oxidative stress by detoxifying ROS to hydrogen peroxide (Zai, et al., 2010). Reducing oxidative stress makes this gene an important player in the NRF2 protective response.

Another phase II detoxification gene transcribed by activated NRF2 is heme oxygenase 1 (HMOX1). HMOX1 codes for heme oxygenase 1 enzyme, which mediated the first step in heme catabolism of biliverdin (Poss & Tonedawa, 1997). Biliverdin is an antioxidant product important to the cellular stress response (Jansen & Daiber, 2012). HMOX1 mutants are shown to

have a decreased response to stress and are unable to protect cells from oxidative damage (Poss & Tonedawa, 1997). Activating this system via NRF2 is an important component of the proposed protective mechanism of NRF2 activation.

Glutathione S-transferases (GST) are also transcribed by NRF2 and have important detoxification properties (Chanas, et al., 2002) (Morimitsu, et al., 2002). The GST family of enzymes functions to detoxify electrophiles by glutathione conjunction (Strange, Spiteri, Ramachandran, & Fryer, 2001). This provides protection against ROS. GST can also act as a cofactor with other antioxidant products to enhance oxidative stress reduction (Lushchak, 2012).

The ability of NRF2 to upregulate these antioxidant products is key to NRF2 activation as a mechanism of protection. If NRF2 activation can be intentionally induced by a supplement, such as sulforaphane or 6-HITC, then targeted therapies for the reduction of toxicant exposure and associated oxidative stress can be developed. Nrf2 activation as a mechanism of protection is currently being tested in many different applications, including protection from pollutants.

Current Uses of NRF2 Activation

Activation of NRF2 via sulforaphane has been tested as a mechanism of protection against pollutants. Acrolein (2-propenal) is a highly reactive widespread environmental pollutant that provides a health hazard to humans, including atherosclerosis and Alzheimer's disease. Exposure to acrolein comes from ingesting fried foods, as it is produced when oil is over heated. Oxidative stress is the main mechanism through which acrolein induces health issues. Sulforaphane was tested as a protective measure against oxidative damage in peripheral blood mononuclear cells (PBMC). In this study PBMCs were collected from volunteers and the cells were treated with acrolein only or acrolein and sulforaphane. Results of this investigation showed that

sulforaphane was able to reduce the oxidative stress induced by the pollutant. (Qin, Deng, & Cui, 2016)

Sulforaphane, produced by broccoli digestion, has also been tested for protection against air pollution. In He-He Township of Qidong China a 12-week clinical trial investigated the effects of a broccoli rich drink on the detoxification of air pollution. 291 participants were chosen from this region of china where air pollution is high. Air pollution has been associated with lung cancer and cardiopulmonary disease. The broccoli rich drinks contained a daily dose of 40 μmol of sulforaphane. Urinary analysis showed that participants receiving the drink had increased pollutant detoxification compared to participants receiving the placebo. (Egner et al., 2014)

Conclusion

The idea that Nrf2 activation can be used to promote the expression of systems that protect organisms from the deleterious effects of toxic agents or physiological stressors is not new. To truly be able to utilize Nrf2, more characterization of its activators and downstream effects are needed. Nrf2 is a complex gene. It is important to learn more about its activation and downstream mechanisms because it could be an important way, we can protect ourselves from environmental insults.

Furthermore, to be able to utilize NRF2 as a protective supplement more studies need to be done on its effects in various tissues. It has been shown in human tissues that the Nrf2 gene, while widely found, is most highly expressed in the adult muscle, kidney, lung, liver and in fetal muscle (Moi, Chan, Asunis, Cao, & Kan, 1994). This could be because these tissues produce the most ROS through high energy production via oxidative phosphorylation, and NRF2 is needed to

reduce oxidative stress. If this is the case it must be investigated how NRF2 functions in tissues with low ROS. If sulforaphane supplementation reduces healthy levels of ROS, then there could be negative effects with supplementation. Individual tissues must be tested against increased NRF2 activation to ensure supplementation would not cause negative effects.

Time period and developmental stage are also important factors to study further in regard to protective NRF2 activation. NRF2 activation would be most helpful in stages in which the effects of environmental pollutants are most detrimental. Fetal development is a time period that is particularly sensitive to disruption by environmental pollutants (Bernal & Jirtle, 2010). During fetal development many systems are being perfectly coordinated and programmed. Excess oxidative stress caused by environmental pollutant exposure can lead to the development of birth defects and eventual adult disease (Rodríguez-Rodríguez, et al., 2018). Decreasing oxidative stress via an NRF2 activator during prenatal development could prevent birth defects and adult disease. However due to the sensitivity and complexity of fetal development, exposing the fetus to an activator of NRF2 could cause adverse effects and disrupt needed programing induced by ROS levels. Further studies need to be done on the effects of NRF2 activation during fetal development.

Being able to characterize the effects of NRF2 activation in different tissues and at different time periods will better allow for the use of NRF2 activators as protection from environmental chemicals. However, this pathway shows exceptional potential to be a system that can be utilized to protect against environmental pollutants. Using the upregulation of endogenous processes to buffer our systems from environmental insults is key in this continually polluted world.

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How can we reduce the effects of environmental pollutants during prenatal development?

Background and Significance

Exposure to pollutants is unavoidable. We are constantly exposed to a variety of chemicals through inhalation, ingestion, and dermal contact (Lioy, 1990). Many of these have negative impacts on our health (Hanson & Gluckman, 2008). For decades people have studied the effects of and mechanisms through which a variety of exogenous environmental chemicals cause toxicity (Kurzel & Cetrulo, 1981) (Fernandez, et al., 2007). Indeed, exposure to such chemicals can alter physiology, development, neurobiology, and behavior and lead to a variety of disorders (Alias, et al., 2019).

As the endocrine system regulates hormone signaling throughout the body, its dysregulation by pollutants has far reaching effects (DeWitt & Patisaul, 2018) (Fernandez, et al., 2007). In fact, endocrine disrupting chemicals, or EDCs, are a class of pollutants that target the endocrine system (Bergman, Heindel, Jobling, Kidd, & Zoeller, 2012). Disruption to this system, especially during development, can be catastrophic. Endocrine signaling during fetal development is responsible for fetal growth, cellular differentiation and physiology, and interactions with the mother such as nutrient transport (Gude, Roberts, Kalionis, & King, 2004). Therefore, endocrine disruption during development can induce dysregulation of fetal programing, leading to birth defects and adult disease (DeWitt & Patisaul, 2018) (Hanson & Gluckman, 2008) (Fernandez, et al., 2007).

Although, a significant amount of work has identified the effects of chemical pollutants and their mechanisms of toxicity, less work has focused on understanding the mechanisms through which individuals protect themselves or their developing young from contaminants (Heindel, 2018).

For example, the placenta is a transient endocrine organ that grows and develops with the fetus, and thus is a target for EDCs (Yang, Song, & Lim, 2019). It regulates maternal-fetal exchange and produces hormones that sustain pregnancy, detoxifies toxins and toxicants, and regulates oxidative stress responses (Gude, Roberts, Kalionis, & King, 2004). Although the placenta is recognized as an important endocrine organ, and its health is critical for fetal health and development, the role that it plays in protecting the fetus from xenobiotics including EDCs is understudied (Thornburg, Boone-Heinonen, & Valent, 2020). Little is known about how placental health and function could be augmented by prenatal supplements to increase its protective mechanisms.

One protective mechanism found in the placenta (He, et al., 2016), that could be augmented to induce placental protection is the nuclear factor erythroid 2-related factor 2, or Nrf2, pathway. Nrf2 is a gene that encodes for the NRF2 transcription factor which is a part of the basic leucine zipper protein family. NRF2 regulates genes which contain antioxidant response elements (ARE) in their promoters many of which encode proteins involved in detoxification and responses to cellular injury, inflammation, and reactive oxygen species (Kaspar, Niture, & Jais, 2009). Without activation NRF2 is bound by Keap1, a cytosolic inhibitor that exists as a dimer and keeps NRF2 retained in the cytoplasm. If Nrf2 is not activated, Keap1 binds to the Cul3/Rbx complex to induce Nrf2 ubiquitination (Kaspar, Niture, & Jaiswal, 2009).

Sulforaphane is a potent activator of NRF2. It is produced from the metabolism of glucoraphanin, by the enzyme myrosinase, after eating cruciferous vegetables, and has been proposed as an ideal transplacental candidate prenatal supplement to protect the fetus from contaminants (Fahey, et al., 2015) (Philbrook & Winn, 2014) (Jahan, Zahra, Irum, Iftikhar, & Ullah, 2014). Sulforaphane's ability to activate NRF2 and increase mechanisms that both reduce

toxicant exposure via detoxification and heal cytotoxic effects make it an obvious contender for rescuing placental damage caused by EDCS as well as for augmenting the placenta's natural protective mechanisms

Indeed, our laboratory has shown that we can reduce the severity of a common EDC-induced birth defect caused by vinclozolin (a model antiandrogenic EDC) by supplementing exposed pregnant mice with sulforaphane (Amato et al, in prep). The McCoy laboratory has developed a strong model system to determine the mechanisms through which sulforaphane protects the developing fetus from EDCs. We use vinclozolin as a model EDC that competitively binds the androgen receptor and causes oxidative stress (Kavlock & Cummings, 2005) (Gazo L. , Linhartova, Shaliutina, & Hulak, 2013). We have determined the concentrations known to induce hypospadias, a birth defect of the male genitalia, 100% of the time, and have identified the dose of sulforaphane (45mg/kg) that reduces hypospadias incidence and severity at this high concentration of vinclozolin. We then showed that sulforaphane can reduce the potency (diminish the dose response) of vinclozolin. We then showed, via a knockout study, that sulforaphane's protective mechanisms are dependent on the presence of NRF2 (Amato et al, in prep.)

The ability of sulforaphane to rescue genitalia development lends confidence to the idea that sulforaphane could protect the placenta from environmental insults such as vinclozolin exposure. Importantly for this work, vinclozolin exposure is known to affect placental health and changes healthy placental physiology. For example, placental vascularization at the site of maternal fetal exchange is reduced by exposure to vinclozolin during pregnancy (Koçkaya, Süloğlu, Karacaoğlu, & Selmanoğlu, 2014). Although, vinclozolin is a model EDC and is convenient to use in laboratory studies, placental health is also affected by other xenobiotics. For example,

bisphenol A and para-nonylphenol, decrease the expression of the ABCG2 transporter, a protective protein, in the human placenta (Sieppi, et al., 2016). Additionally, it has been widely observed that exposure to environmental pollutants increases oxidative stress in the placenta which has been shown to lead to conditions such as preterm birth, intrauterine growth restriction, and program adult disease (Thornburg, Boone-Heinonen, & Valent, 2020) (Manikkam, Tracey, Guerrero-Bosa, & Skinner, 2013). Because placental health is being compromised by environmental pollutants, developing a prenatal supplement that restores the health of the placenta and protects the developing fetus would be invaluable.

This research will determine the molecular mechanisms of toxicity and protection that occur within the placenta due to EDC exposure, and will test the hypothesis that we can augment protective pathways with prenatal supplementation of sulforaphane and reduce the molecular changes caused by vinclozolin. We also determine the molecular changes that occur with exposure to sulforaphane alone (without toxicants). Importantly the protective pathways that occur in the placenta could be helping to protect the fetus from vinclozolin and playing an important role in the sulforaphane-induced rescue of sexual development that we consistently find in animals exposed to both vinclozolin and sulforaphane relative to vinclozolin alone.

Methods

Experimental Model

To test the hypothesis that exposure to vinclozolin, a model anti-androgenic EDC, alters placental gene expression, and that sulforaphane restores vinclozolin altered gene expression to baseline levels, male and female mice heterozygous for Nrf2 (cross of C57BL/6J Stock No: 000664 crossed with B6.129X1-Nfe2l2tm1Ywk/J Stock No: 017009 from Jackson Laboratories)

were time mated. This crossing design was used so that pregnant dams would, on average, give birth to both wild type (WT) and knock out (KO) brothers. This study, however, focuses on wild type individuals only. Females were checked for vaginal plugs each morning and presence of a plug indicated that mating had occurred the previous night. Embryos of females with vaginal plugs were scored as being embryonic day (E) at noon of the day the plug was identified. Impregnated females were exposed to either corn oil (CO, control), vinclozolin (V) only, sulforaphane (S) only, or vinclozolin + sulforaphane (SV) starting on E13.5 through E16.5. One hour after the last treatment dose on E16.5 dams were humanely sacrificed. Embryos were removed from the uterus, a tail snip was taken for genotyping, and placentas were collected, weighed, frozen on liquid nitrogen, and stored at -80C until they were processed for transcriptomics and proteomics. PCR was conducted to determine the genotype of every individual. Dam is treated as the unit of replication.

Transcriptomics

For transcriptomics analyses one wildtype male placentas per dam (N=5 for CO, S, SV; N=6 for V) were submitted to the North Carolina State Genomic Sciences Laboratory (Raleigh, NC, USA) for RNA extraction, Illumina RNA library construction, and sequencing. Total RNA was extracted from tissues using a Qiagen TissueLyser bead homogenizer in conjunction with a Qiagen RNeasy mini kit according to the kit-provided protocol and eluted in nuclease-free water prior to RNA library construction. Purification of messenger RNA (mRNA) was performed using the oligo-dT beads provided in the NEBNExt Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, USA). Complementary DNA (cDNA) libraries for Illumina sequencing were constructed using the NEBNext Ultra Directional RNA Library Prep Kit (NEB) and NEBNext Mulitplex Oligos for Illumina (NEB) using the manufacturer-specified protocol.

Briefly, the mRNA was chemically fragmented and primed with random oligos for first strand cDNA synthesis. Second strand cDNA synthesis was then carried out with dUTPs to preserve strand orientation information. The double-stranded cDNA was then purified, end repaired and "a-tailed" for adaptor ligation. Following ligation, the samples were selected a final library size (adapters included) of 400-550 bp using sequential AMPure XP bead isolation (Beckman Coulter, USA). Library enrichment was performed and specific indexes for each sample were added during the protocol-specified PCR amplification. The amplified library fragments were purified and checked for quality and final concentration using an Agilent 2200 Tapestation with a High Sensitivity DNA chip (Agilent Technologies, USA) and a Qubit fluorometer (ThermoFisher, USA). The final quantified libraries were pooled in equimolar amounts for clustering and sequencing on an Illumina NovaSeq 6000 DNA sequencer, utilizing a single S4 150x2 bp paired-end sequencing reagent kit with XP workflow (Illumina, USA). The software package Real Time Analysis (RTA), was used to generate raw bcl, or base call files, which were then de-multiplexed by sample into fastq files for data submission.

RNA-Seq Data Analysis

Data analysis was performed in consultation with Bioinformatics Core at NCSU Center for Human Health and the Environment. We generated on average ~37 million paired-end reads of raw RNAseq data for each replicate with a length of 150 bp. The quality of sequence data was assessed using FastQC command line application and 12 poor quality bases were trimmed from the 5'-end. The remaining good quality reads were aligned to the mouse reference genome (mm38 version 87) using STAR aligner (Love et al., 2014). For each replicate, per-gene counts of uniquely mapped reads were using htseq-count script from the HTSEQ python package (version 0.11.1). Count data were imported to R statistical computing environment (The R

Development Core Team, 2017). Data normalization and analysis were conducted using DESeq2 Biocoductor package (Love and others, 2014). We fitted a linear model using the treatment levels and differentially expressed genes were identified before and after applying multiple testing correction using Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995; p-adjust < 0.05). During broad investigations where we investigate patterns and pathways an uncorrected p-value < 0.05 was considered statistically significant, and when we identify specific genes as biomarkers, a corrected p-value, p-adjust < 0.05, was considered statistically significant.

Directed Comparisons and Functional Analysis

To create a list of differentially expressed genes for each treatment, the expression of each gene within each treatment was compared to its expression in the CO control (p-value < 0.05). These focal treatment contrasts were then compared across treatments so that we could utilize the overlap and relationships between the differentially expressed lists to identify groups of genes as altered due to vinclozolin exposure, groups of genes altered by vinclozolin exposure that were rescued, the genes altered by sulforaphane alone, and candidate mechanisms of resilience. These lists of differentially expressed genes were compared via a three comparison Venn diagram. To calculate and create all Venn diagrams, Bioinformatics & Evolutionary Genomics' webtool, Venn Diagram, was used (Bioinformatics & Evolutionary Genomics, n.d.).

Functional analysis of identified gene groups was done using DAVID Functional Annotation Tool (Huang , Sherman , & Lempicki, Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources, 2009) (Huang , Sherman BT, & Lempicki, Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large

gene lists, 2009). For the functional analysis, the background was set as all 16,657 genes that were detected as good quality reads and the identified gene groups served as the gene list.

Proteomics

Proteomics analysis was conducted on one male placenta per dam (N=5 for CO, V, and SV; N=4 for S) per treatment. Placentas were prepared following published protocols. Briefly, the tissue was homogenized, proteins isolated and enzymatically digested using trypsin, and filtered prior to injection onto the nano-LC/MS (Wiśniewski , Zougman, Nagaraj, & Mann, 2009). A 1 μg of peptide mixture was loaded on the Thermo EASY nano-LC column and separated along a linear gradient to maximize peak capacity and protein identifications (Hsieh, Bereman, Durand, Valaskovic, & MacCoss, 2013). The Q-Exactive Plus mass spectrometer acquired data in positive ion mode and MS/MS data was acquired using a data dependent acquisition for the top 20 most abundant precursor ions.

Proteins were identified using Protein Discoverer 2.1. Relative protein quantitation was performed using label free methods (integration of area under the curve) using Skyline and the MSstats package. Each individual peptide was manually evaluated to ensure retention time reproducibility, high dot product (>0.8), and proper integration boundaries. Normalization was performed to a spiked exogenous protein standard.

Transcriptomics to Proteomics Comparisons

To refine the list of candidate mechanisms of resilience we quarry the proteome for products of the genes we identified as such. To directly compare transcriptome and proteome data, Uniprot accessions used in proteomic data were converted to Ensembl Stable IDs using DAVID gene

conversion tool (Huang , Sherman , & Lempicki, Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources, 2009) (Huang , Sherman BT, & Lempicki, Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists, 2009). The gene list was compared via Venn diagram to the converted proteomic data set (Bioinformatics & Evolutionary Genomics, n.d.). Overlap between the two groups identifies the genes in the candidate mechanisms of resilience list that have associated protein products. Functional analysis was conducted on the group of overlapping genes using DAVID functional annotation tool referenced above. These functions and pathways are identified as those that are most likely contributing to the resilience to or protection from vinclozolin.

Results

How many genes are differentially expressed from CO in each treatment?

To assess how the treatments affected gene expression, each treatment was statistically compared to CO (p-values 0.05). V caused 1002 genes to be differentially expressed, whereas S changed expression of only 205 genes relative to CO. When vinclozolin and sulforaphane were given together the SV treatment changed 694 genes relative to CO. Therefore, adding sulforaphane to the vinclozolin treatment led to a reduction in the total number of affected genes relative to vinclozolin alone by 30.7%. The identity of genes that are differentially expressed relative CO listed here depend on treatment.

To further understand the nature of the gene expression changes within and among treatments and allow for more thorough interpretation of the unique and overlapping changes, all three of the focal contrasts were compared via Venn diagram (Figure 1). There were 776 genes differentially expressed from CO that were unique to V exposure (Figure 1, a.). These 766 genes

are considered effects of vinclozolin exposure. There were 159 genes that were differentially expressed from CO in both V and SV treatments (Figure 1, b.) These genes are considered effects of V exposure that are not corrected with S supplementation. There were 102 genes that were uniquely different from CO due to S supplementation (Figure 1, c) and identify the gene affected by S when no pollutant is present. There were 42 genes that were differentially expressed from CO in both V exposure and S supplementation (Figure 1, d.). These 42 are considered effects of an exogenous chemical exposure that are no longer different in the SV treatment (so the combination treatment leads to no change in these genes). Thirty-five genes were differentially expressed from CO in all treatments (Figure 1, e.). These genes are considered effects of a new chemical introduction which are not ameliorated by sulforaphane supplementation. The 474 genes that are differentially expressed from CO and are unique to SV (Figure 1, f.) are considered candidate mechanisms of resilience as they are the genes with novel expression profiles specific to the treatment (VS) which is known to reduce vinclozolin induced birth defects (Amato et al., in prep). There are 26 genes that are differentially expressed from CO in both S and SV (Figure 1, g.). These genes are considered effects of S supplementation with or without the presence of a pollutant and are also considered candidate mechanisms of resilience. One particularly interesting gene in this gene group is Cyp1a1. This is an important xenobiotic-metabolizing enzyme of the placenta (Stejskalova & Pavek, 2011). Although Cyp1a1 was differentially expressed from CO in both SV treatment and S treatment, its expression was upregulated in SV treatment while downregulated in the S treatment. This is the only differentially expressed protein that was overlapping between two treatments yet expressed in different directions relative to CO. All other differentially expressed proteins were different from CO in the same direction.

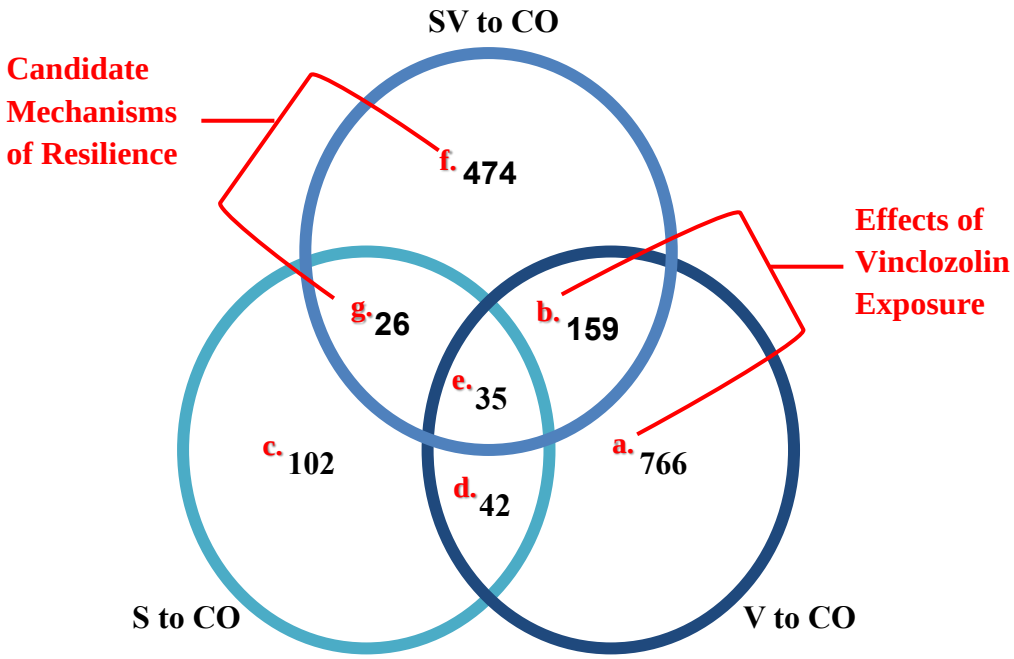


Figure 1: Comparison of differentially expressed from CO across treatments. To understand the overlap between the groups of genes that are differentially expressed from CO in each treatment, all three comparisons were compared via Venn diagram. This allowed for the identification of discrete groups with specific overlap patterns.

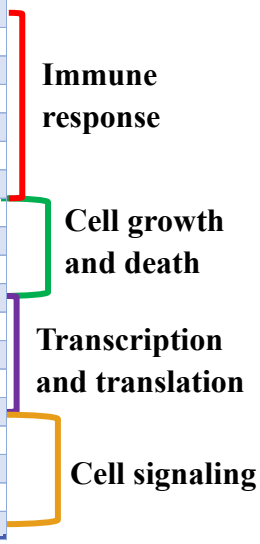
What are the biomarkers of V exposure and which are corrected by S supplementation?

Next, we wanted to identify individual genes that were biomarkers of exposure to V (affected by V exposure) and investigate which of these genes have normalized or rescued expression levels when sulforaphane is crossed with vinclozolin. To reduce the false positives associated with type I error which is inflated by multiple comparisons, we used a higher threshold of significance (adjusted p-value < 0.05) in this comparison. We found 19 genes that were significantly affected by vinclozolin and were identified as biomarkers of exposure. We then asked which of these genes were no longer significantly different in the SV to CO comparison and found that none of the 19 vinclozolin-induced gene expression differences were different from CO in animals exposed to SV (Table 1). In fact, at this level of significance (adjusted p-value < 0.05) there are

no genes that were differentially expressed from CO in the SV, or S treatments. The 19 biomarkers of vinclozolin exposure fell into four main functional categories, immune response, cell growth and death, transcription and translation, and cell signaling. We formally evaluated these functions for enrichment in the gene list using DAVID functional annotation tool below.

Table 1: Biomarkers of vinclozolin exposure. *19 genes were identified as biomarkers of vinclozolin exposure from comparison of V to CO at FDR 0.05. These 19 genes fall into four functional categories, immune response, cell growth and death, transcription translation, and cell signaling. There is some overlap between the categories as some genes contribute to multiple functions.*

Gene name	V/CO	Gene Involvement	Refrence	VS/CO
Zbp1	down	Detection of foreign DNA	Rothan et al, 2019	nsd
Mpeg1	down	Response to bacterial infection	McCormack et al, 2015	nsd
Ccr5	down	Receptor for chemokines on white blood cells	Behbahani et al, 2000	nsd
Iigp1	down	Resistance to intracellular pathogens	Martens et al, 2005	nsd
Oasl2	down	Inate cellular antiviral respose	Eskildsen et al, 2003	nsd
Rtkn2	up	White blood cell formation	Collier et al, 2004	nsd
Irgm1	down	Inate immune response in regulation of autophagy	Azzam et al, 2017	nsd
Ifi204	down	Cell growth inhibition via p53/TP53	Johnstone et al, 2000	nsd
Iqcm	up	Function unknown, seen in some cancer cells	Pleasance et al, 2010	nsd
Casp1	down	Execution phase of cell apoptosis	Kapplusch et al, 2019	nsd
Asf1a	up	DNA replication	Mousson et al, 2005	nsd
Parp10	down	DNA repair	Chou et al, 2006	nsd
Parp12	down	Stopping translation in response to oxidative stress	Vyas et al, 2014	nsd
Phax	up	RNA translation	Gebhardt et al, 2015	nsd
Uba7	down	Activation of ubiquitin	Kok et al, 1993	nsd
Sgcz	up	Bridging the inner cytoskeleton and the extra-cellular matrix	Rose et al, 2010	nsd
Sntg1	up	Involved in cellular membrane transport	Bashiardes et al, 2004	nsd
Gbp2	down	Hydrolyzing GTP to GDP	Cheng et al, 1991	nsd
Rab36	down	Protein transport	Mori et al, 1999	nsd



What functions and processes are associated with the corrected biomarkers of exposure?

Molecular function analysis of the 19 biomarkers of vinclozolin exposure returned three functions as being significantly enriched (Figure 2, A). GTP binding, GTPase activity, and NAD+ ADP ribotransferase activity are downregulated in the V treatment group. The genes present in the gene list associated with GTP binding and GTPase activity were Gpb2, Iigp1, and Irgm1. Rab36 was the only gene not associated with both functions, and was associated with GTP binding. Enrichment of NAD+ ADP ribotransferase activity was driven by Parp10 and Parp12. The 19 biomarkers of vinclozolin exposure were enriched for seven biological processes

each of which was reduced relative to the CO treatment (Figure 2, B). These downregulated processes were cellular response to interferon-beta, innate immune response, immune system process, positive regulation of interleukin-1 beta secretion, defense response to protozoan, defense response, and regulation of autophagy. The genes present on the gene list associated with cellular response to interferon-beta were Gbp2, Irgm1, Ifi204, and Iigp1. Enrichment of innate immune response and immune system process was caused by Oasl2, Zpb1, Irgm1, and Iigp1. The genes responsible for the enrichment of positive regulation of interleukin-1 beta secretion were Casp1 and Ccr5. Enrichment of defense response to protozoan was caused by Gpb2 and Iigp1. The genes responsible for enrichment of defense response were Ccr5 and Irgm1. Enrichment of regulation of autophagy was caused by Casp1 Iigp1. Kegg pathway analysis returned two pathways as being significantly enriched in the differentially expressed list (Figure 2, C). Cytosolic DNA sensing pathway and toxoplasmosis (immune) both of which are

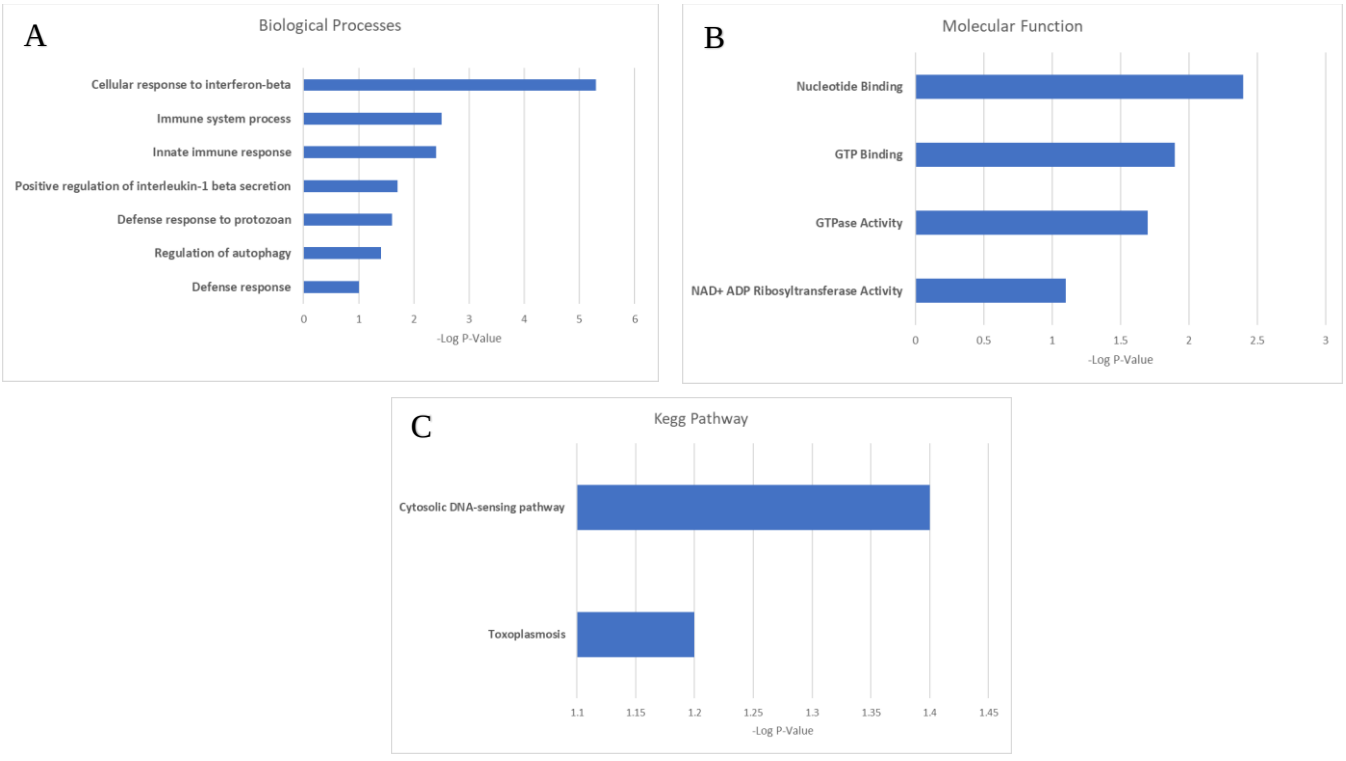


Figure 2: DAVID pathway and process analysis*. The DAVID functional annotation tool was used to identify biological processes (A), molecular functions (B), and kegg pathways (C) that were enriched in the group of vinclozolin exposure biomarkers. This figure does not indicate directionality of function changes but rather strength of enrichment and therefore confidence in correct identification.*

downregulated in the V treatment group relative to CO. The genes present in the gene list associated with Cytosolic DNA sensing pathway were Zbp1 and Casp1. The genes responsible for the enrichment of toxoplasmosis pathway were Ccr5 and Irgm1. No genes were differentially expressed in the SV to CO comparison at a confidence threshold of adjusted p-value of 0.05, therefore we assume these functions and pathways remain unaffected in the SV treated animals.

What are the candidate mechanisms of resilience?

To understand what genes and mechanisms within the SV treatment group are driving the correction of vinclozolin affected genes, functional and pathway analysis was conducted on the 500 (Figure 1, f + g) genes identified as candidate mechanisms of resilience. These genes are considered candidate mechanisms of resilience because their expression was unchanged in the V to CO comparison (vinclozolin did not alter them) but was altered in the SV to CO comparison the treatment ($p < 0.05$) that lead to a reduction in the number of genes affected by vinclozolin from 1002 to 194 (figure 1, a + b + d + e =1002 to b +e=194). Therefore, the number of genes effected by vinclozolin were reduced by 80.6% in the SV treatment. Also, these genes are differentially expressed in the SV treatment compared to CO, showing they are altered in response to an exogenous exposure. Molecular function analysis of these 500 genes returned functions related to oxidative phosphorylation (ex. oxidoreductase activity) and cell signaling (voltage-gated ion channel activity) (Figure 3, A). The biological processes associated with resilience related to fetal development (ex. sprouting angiogenesis) and immune response functions (regulation of inflammatory response) (Figure 3, B). Pathway analysis showed five pathways associated with chemical resilience (Figure 3, C). These pathways are consistent with the molecular functions and biological processes associated with vinclozolin resilience. For example, the Parkinson's disease pathway includes the ubiquitin pathway (cell signaling) and

mitochondrial pathway (oxidative phosphorylation), while the intestinal immune network for IgA production encompasses many immune response processes (Figure 3, A, B, C).

Figure 3: DAVID analysis on candidate mechanisms of resilience. *The DAVID functional annotation tool was used to identify biological processes (A), molecular functions (B), and kegg pathways (C) enriched in the group of candidate mechanisms of resilience. Candidate mechanisms of resilience were identified in the comparison of V to CO vs SV to CO (Figure 1, B, r). These 500 genes were identified as candidate mechanisms of resilience because they are differentially expressed from CO in the rescued treatment (SV) but not differentially expressed in the V exposed treatment. This shows these genes are an active response to the SV condition. Analysis showed enrichment of several processes associated with oxidative stress, oxidative phosphorylation, and immune response.*

What protein products are associated with the candidate mechanisms of resilience?

To refine the list of molecular mechanisms driving resilience and determine the pathways that were most likely responsible for reducing the effects of vinclozolin, we matched the candidate resilience genes to their associated protein products. A Venn diagram was used to compare the 500 genes identified as candidate mechanisms of resilience and the differentially expressed

proteins in the SV to CO comparison (Figure 4). The presence of protein products from the candidate mechanisms of resilience would indicate a much higher confidence that these genes and their associated proteins are likely the mechanisms that are causing the rescue of gene expression changes. There were 5 genes from the candidate mechanisms of resilience gene group that showed associated gene products from the proteomics dataset (Figure 4).

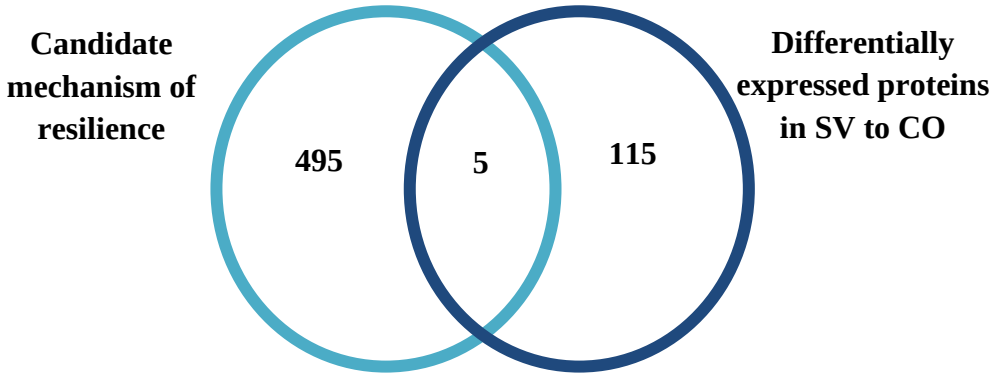


Figure 4: Identification of protein products associated with candidate mechanisms of resilience via Venn diagram comparison. *Gene group identified as candidate mechanisms of resilience (Figure 1, f +g) were compared to the differentially expressed proteins in the SV to CO comparison from the converted protein list in order to identify a list of genes strongly related to the rescue seen in the SV treatment.*

The 5 proteins and the genes they correspond with are listed in Table 2. Gene expression, identified from RNA transcript, was identified at the same time point as protein expression (E16.5). Proteins come from translation of RNA transcript, but not all transcript codes for a functional protein. Functional analysis with DAVID functional annotation tool reveled

Table 2: Proteins associated with candidate mechanisms of resilience genes*. Five genes were identified when candidate mechanisms of resilience genes were compared to differentially expressed proteins in the SV to CO comparison. These represent candidate mechanisms of resilience with associated protein products.* ***Green*** *text represents increased expression relative to CO while* ***red*** *text represents decreased expression relative to*

Uniprot Acession	GeneName	Protein
P54071	Idh2	Isocitrate dehydrogenase 2 (NADP+), mitochondrial
Q61655	Ddx19a	ATP-dependent RNA helicase
P03930	mt-atp8	ATP synthase F0 subunit 8
P05480	Src	Neuronal proto-oncogene tyrosine-protein kinase
P21278	Gna11	Guanine nucleotide-binding protein subunit alpha-11

enrichment for one biological process and two kegg pathways. The biological process enriched in this protein list was positive regulation of apoptotic process. The proteins present on the protein list that are associated with positive regulation of apoptotic process are ATP-dependent RNA helicase and neuronal proto-oncogene tyrosine protein kinase. The kegg pathways enriched in this protein list were GnRH signaling pathway and gap junction. Both pathways were associated with neuronal proto-oncogene tyrosine protein kinase and guanine nucleotide-binding protein subunit alpha-11 from the protein list.

Discussion

We have found that prenatal supplementation with sulforaphane can reduce gene expression changes caused by vinclozolin (V) exposure. V exposure induced 1002 genes to be altered from CO and when sulforaphane was supplemented with vinclozolin (SV) only 194 of those genes were still altered (Figure 1). In fact, there is an 80.6% overall reduction in the number of differentially expressed genes from CO in the SV treatment. As CO expression is inherently considered normal, SV treatment normalized over 80% of the genes that vinclozolin made abnormal. This correction is also seen in the identified biomarkers of vinclozolin exposure. None of the biomarkers of vinclozolin exposure are seen differentially expressed in the SV treatment. In fact, there are no genes that are differentially expressed from CO in the SV treatment at the higher confidence (adjusted p-value) threshold. The correction of these vinclozolin effected genes by sulforaphane show that prenatal sulforaphane can protect the placenta and therefore the fetus from environmental pollutants.

To present sulforaphane as a general prenatal supplement, it must be verified that S alone treatment does not induce a high instance of gene expression changes. Venn diagram comparison

of genes differentially expressed from CO in each treatment revealed, S treatment induced gene expression changes relative to the CO control, but it did not cause nearly as many changes as V exposure (Figure 1, A). This suggests that sulforaphane alone is less likely to induce physiological changes that induce poor health. Indeed, in our previous study individuals exposed to sulforaphane alone did not have any birth defects or suffer any noticeable health defects (Amato et al., in prep). Additionally, at the adjusted p-value threshold there were no genes differentially expressed from CO in the S alone treatment. The normal gene expression profile at this confidence level also suggests that sulforaphane exposure alone is not responsible for major gene expression changes that could be considered harmful.

While the overall rescue that sulforaphane supplementation induces is important, it is also important to look at specific gene changes that SV induces to understand what effects the treatment is having. The 26 genes that were differentially expressed in both S and SV treatments but not in V exposure are associated with sulforaphane exposure and are not specific to or modified by the addition of V to S with one exception. Cyp1a1 expression is increased relative to CO in the SV treatment while it is down regulated relative to CO in the S treatment. Cyp1a1 codes for the protein Cytochrome P450 1A1, a member of the cytochrome P450 family, and is an important xenobiotic-metabolizing enzyme of the placenta (Stejskalova & Pavek, 2011). Increased expression in the SV treatment shows that sulforaphane's effect on this gene is dependent on the environment, or V exposure, and a mechanism of rescue from pollutant exposure. Cyp1a1 expression is not differentially expressed from CO in the V exposure treatment, so V alone does not cause its expression to increase. This is interesting because upregulation of this gene in the presence of a pollutant would be an appropriate adaptive response. It appears, however, that the placenta does not upregulate Cyp1a1 in the presence of V

unless we supplement with sulforaphane (SV) which facilitates the V exposed placenta to respond to the pollutant. This is an especially important correction given the importance of Cyp1a1 to placental response to environmental pollutants (Stejskalova & Pavek, 2011).

The19 genes identified as biomarkers of vinclozolin exposure were associated with several functions that are known to be important for healthy placenta function. One particularly concerning change caused by vinclozolin exposure was the downregulation of Parp12. Parp12 is involved with stopping translation in response to oxidative stress (Welsby, et al., 2014). Downregulating this process in the presence of a pollutant that increases oxidative stress (Gazo I. , Linhartova, Shaliutina, & Hulak, 2013) is evidence of a maladaptive response. Indeed, such maladaptive responses might be quite common as animal models and humans have likely not evolved adaptive mechanisms to detect synthetic chemicals and activate protective mechanisms. If we can augment these endogenous and natural protective mechanisms with sulforaphane or some other protective supplement we should be able to protect our tissues and developing embryos from contaminants. Indeed, expression of Parp12 in the SV treatment placentas was expressed at normal (control) levels. We can see that S in the context of V (SV) alters a variety of genes that are specific to that environment (treatment) and are associated with reductions in biomarkers of vinclozolin exposure (Table 1), so S facilitates adaptive responses that are not elicited by vinclozolin alone. The correction of these biomarkers and all gene expression changes at this threshold (adjusted p-value) suggest that sulforaphane supplementation is restoring gene expression changes to normal levels and promotes placental health.

The next step in establishing sulforaphane as a prenatal therapy to protect the placenta from pollutants is understanding the mechanisms through which sulforaphane is inducing its rescue effect (e.g., reductions in vinclozolin-induced gene expression). We identified 500 genes as

candidate mechanisms of resilience because they were differentially expressed from CO in the SV (rescued) treatment but not differentially expressed in the V exposed treatment. This shows that these genes are affected by S exposure in a context that is specific to an environment that includes a toxicant. Functional analysis returned several processes associated with oxidative stress, oxidative phosphorylation, and immune response as being significantly enriched in the 500 candidate mechanisms of resilience and are functions important to the rescue effect.

Although much work has focused on understanding the gene expression changes that occur due to contaminant exposure, we know that gene expression profiles do not match with their functional protein counterparts. To increase the strength of our inference we refined our candidate mechanisms of resilience to include only the protein products of the 500 candidate genes that were differentially expressed in the SV treated placentas. Linking the genetic response to a protein product facilitates confidence in the pathway as a directed action that is specific to the SV treatment and rescue effects that we find here and in previous studies (Amato et al, in prep).

The five genes that are candidate mechanisms of resilience had associated protein products that were also differentially expressed in the SV treatment suggest that sulforaphane supplementation is working to decrease oxidative stress, regulation of ATP production (oxidative phosphorylation) and cell signaling (Table 2). Isocitrate dehydrogenase 2 (Idh2) is a mitochondrial enzyme whose main function is NADP+-dependent oxidative decarboxylation of isocitrate to 2OG (Smolková & Ježek, 2012). It has been shown that upregulation of Idh2 buffers from the effects of increased reactive oxygen species and oxidative stress (Sup Kil & Park, 2005). Also buffering against oxidative stress, ATP-dependent RNA helicase has been shown to reduce the formation of stress granules, which are condensed non-translating RNA (Mikhailova,

et al., 2017). Stress granule formation is induced by conditions of oxidative stress (Chen & Liu, 2017). Idh2 and Ddx19a protein expression and gene expression was increased in the SV treatment suggesting that one mechanism that supplementation with sulforaphane induces its rescue is by decreasing oxidative stress. ATP synthase F0 subunit 8 is a small hydrophobic component of the membrane associated F0 sector of ATP synthase, which functions in the production ATP via oxidative phosphorylation (Devenish, Papakonstantinou, Galanis, & Law, 1992). mt-atp8 protein expression is increased relative to CO in the SV treatment while mt-at8 gene expression is downregulated relative to CO in the SV treatment (Table 2). Since transcriptomic and proteomic datasets were taken at the same developmental day and dosing time frame, this suggests that some mechanism of feedback is taking place to regulate oxidative phosphorylation. Protein expression is down compared to normal (CO), so gene expression is increased compared to normal for eventual compensation. Functional analysis of these five genes showed neuronal proto-oncogene tyrosine-protein kinase and guanine nucleotide-binding protein subunit alpha-11 being associated with GnRH signaling pathway and gap junction pathways. GnRH signaling pathway has also been associated with regulating the stress response (Li, Mao, & Wei, 2008). Gap junction pathways have been shown to play a role in placental development and the cell differentiation process (Malassiné & Cronier, 2005). Decreasing oxidative stress, regulating oxidative phosphorylation, and cell signaling are important processes for the health of the placenta, and the regulation of these processes by supplementation of sulforaphane are important to the mechanism of resilience for protection against pollutants.

Further research is required to determine how these genes and proteins form complex networks to drive resilience to toxicants, and to understand how the biological processes that we have identified are specifically related to resilience. The present study, however, takes an important

step toward helping to develop sulforaphane as a protective prenatal supplement that will reduce the effects of environmental toxicants on placental gene expression and function. Together with our previous work, and that of others, this study provides further evidence that sulforaphane is an ideal supplement to protect the placenta and fetus from various pollutant exposures, as many pollutants induce changes that are likely to be corrected by these general mechanisms (Jahan, Zahra, Irum, Iftikhar, & Ullah, 2014) (Lioy, 1990) (Kurzel & Cetrulo, 1981)

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Appendix A

IACUC Explanation Statement

No animal was handled for the completion of this study. The tissue data used for this study was collected during a previous study in the McCoy laboratory.