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## XENOESTROGEN-SPECIFIC MECHANISMS OF DEVELOPMENTAL REPROGRAMMING CORRELATE WITH GENE EXPRESSION AND TUMOR DEVELOPMENT

Kristen L. Greathouse

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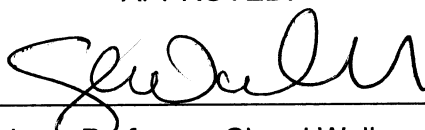
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DEVELOPMENT


by

Kristen Leigh Greathouse, B.S., M.S.

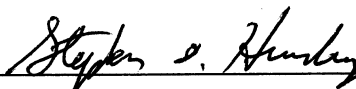
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**XENOESTROGEN-SPECIFIC MECHANISMS OF DEVELOPMENTAL  
REPROGRAMMING CORRELATE WITH GENE EXPRESSION AND TUMOR  
DEVELOPMENT**

A

DISSERTATION

Presented to the Faculty of  
The University of Texas  
Health Science Center at Houston  
and  
The University of Texas  
M. D. Anderson Cancer Center  
Graduate School of Biomedical Sciences  
in Partial Fulfillment  
of the Requirements  
for the Degree of  
DOCTOR OF PHILOSOPHY

by

Kristen Leigh Greathouse, B.S., M.S.  
Houston, Texas

May, 2010

## **Dedication**

I dedicate this dissertation to my marry band of friends who sailed with me on this journey through the tsunamis of crushing failure, the caves of despair, past the sirens of procrastination, to find a sea of tranquility. And a better group of souls I could not have found.

To my parents for their lessons of hard work and humility, which kept me afloat in tempests and moving forward when there was no wind in my sails.

To my husband, my compass and my hero, who daily bolstered my mast of confidence when it was in tatters and shone bright like the sun when storm clouds threatened to hinder my journey.

## **Acknowledgements**

I would like to acknowledge my mentor Dr. Cheryl Walker for sharing her insight and wisdom of what it takes to excel in science. She always challenged me to think critically and most importantly gave me the skills to become a successful scientist.

I would also like to thank all those on my committee, Dr. Stephen Hursting, Dr. David Johnson and Dr. Rick Wood for guidance and support. Their wonderful suggestions and questions kept my project and me on task and allowed me to see it through to completion.

I would finally like to thank all of those lab members past and present who provided me with intellectual and emotional support on a daily basis. Their assistance was invaluable in the success of this work.

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REPROGRAMMING CORRELATE WITH GENE EXPRESSION AND TUMOR  
DEVELOPMENT

Publication No. \_\_\_\_\_

Kristen Leigh Greathouse, Ph.D.

Supervisory Professor: Cheryl L. Walker, Ph.D.

Environmental exposures during sensitive windows of development can reprogram normal physiological responses and alter disease susceptibility later in life in a process known as developmental reprogramming. We have shown that neonatal exposure to the xenoestrogen diethylstilbestrol (DES) can developmentally reprogram the reproductive tract in genetically susceptible Eker rats giving rise to complete penetrance of uterine leiomyoma. Based on this, we hypothesized that xenoestrogens, including genistein (GEN) and bisphenol A (BPA), reprogram estrogen-responsive gene expression in the myometrium and promote the development of uterine leiomyoma. We proposed the mechanism that is responsible for the developmental reprogramming of gene expression was through estrogen (E2)/ xenoestrogen induced-rapid ER signaling, which modifies the histone methyltransferase Enhancer of Zeste homolog 2 (EZH2) via activation of the PI3K/AKT pathway. We further hypothesized that there is a xenostrogen-specific effect on this pathway altering patterns of histone modification, DNA methylation and gene expression. In addition to our novel finding that E2/DES-induced phosphorylation of EZH2 by AKT reduces the levels of H3K27me3 *in vitro* and *in vivo*, this work demonstrates *in vivo* that a brief neonatal exposure to GEN, in contrast to BPA, activates the PI3K/AKT pathway to regulate EZH2 and decreases H3K27me3 levels in the neonatal uterus. Given that H3K27me3 is a repressive mark that has been shown to result in DNA methylation and gene silencing we investigated the methylation of developmentally reprogrammed genes. In support of this evidence, we show that neonatal DES exposure in comparison to VEH, leads to hypomethylation of the promoter of a developmentally reprogrammed gene, *Gria2*, that become hyper-responsive to estrogen in the adult myometrium indicating

that DES exposure alter gene expression via chromatin remodeling and loss of DNA methylation. In the adult uterus, GEN and BPA exposure developmentally reprogrammed expression of estrogen-responsive genes in a manner opposite of one another, correlating with our previous data. Furthermore, the ability of GEN and BPA to developmental reprogram gene expression correlated with tumor incidence and multiplicity. These data show that xenoestrogens have unique effects on the activation of non-genomic signaling in the developing uterus that promotes epigenetic and genetic alterations, which are predictive of developmental reprogramming and correlate with their ability to modulate hormone-dependent tumor development.

## TABLE OF CONTENTS

	PAGE
<b>Approval Page</b>	<b>i</b>
<b>Title Page</b>	<b>ii</b>
<b>Dedication</b>	<b>iii</b>
<b>Acknowledgement</b>	<b>iv</b>
<b>Abstract</b>	<b>v</b>
<b>Table of Contents</b>	<b>vii</b>

## TABLE OF CONTENTS

<b>List of Figures .....</b>	<b>x</b>
<b>List of Tables.....</b>	<b>xi</b>
<b>List of Appendices.....</b>	<b>xii</b>
<b>Chapter 1: Introduction and Background .....</b>	<b>1</b>
<b>1.1 Introduction .....</b>	<b>2</b>
<b>1.2 Developmental Reprogramming .....</b>	<b>2</b>
1.2.1 Endocrine Disruptors .....	3
<b>1.3 Mechanisms of Developmental Reprogramming .....</b>	<b>15</b>
1.3.1 Xenoestrogen-Induced Genomic Mechanisms of Endocrine Disruption .....	16
1.3.2 Xenoestrogen-Induced Non-Genomic Mechanisms of Endocrine Disruption.....	18
1.3.3 Epigenetic Developmental Reprogramming .....	22
1.3.4 Xenoestrogen-Mediated Epigenetic Reprogramming.....	25
1.3.5 Xenoestrogen-Induced Transgenerational Inheritance.....	27
<b>1.4 Xenoestrogens and Developmental Origins of Hormone-Dependent Tumors .....</b>	<b>28</b>
1.4.1 Etiology of Uterine Leiomyoma .....	30
1.4.2 Rodent Uterus Development and the Eker Rat Model of Uterine Leiomyoma .....	32
<b>Chapter 2: Identification of Uterine Leiomyoma Genes Developmentally Reprogrammed by Neonatal Exposure to Diethylstilbestrol .....</b>	<b>38</b>
<b>2.1 Purpose of Study .....</b>	<b>39</b>
<b>2.2 Methodology .....</b>	<b>39</b>
2.2.1 Animals and Treatments .....	39
2.2.2 Tissue Collection and Histology.....	40
2.2.3 Histological Staging of Estrous .....	41

2.2.4 Microarray Analysis.....	41
2.2.5 Microarray and Statistical Analysis .....	42
2.2.6 Microarray Validation and Real-Time PCR (qPCR) .....	42
<b>2.3 Results .....</b>	<b>43</b>
2.3.1 Discovery of Candidate Genes Associated With Uterine Leiomyoma and Developmental Reprograming in Eker Rats.....	43
2.3.2 Developmental Reprogramming of Estrogen-Responsive Genes by Neonatal Exposure to DES .....	51
<b>2.4 Discussion.....</b>	<b>58</b>
<b>Chapter 3: Xenoestrogen-specific modulation of non-genomic signaling through histone methyltransferase, EZH2, correlates with developmental reprogramming of gene expression and tumorigenesis .....</b>	<b>61</b>
<b>3.1 Study Purpose .....</b>	<b>62</b>
<b>3.2 Methodology .....</b>	<b>64</b>
3.2.2 Animals and treatments .....	64
3.2.2 Tissue collection and histological studies .....	65
3.2.3 Histological categorization of estrous .....	65
3.2.4 Real-time PCR (qPCR).....	66
3.2.5 Preparation of tissue lysates and immunoblotting .....	67
3.2.6 Immunohistochemistry.....	68
3.2.7 Immunoprecipitation.....	68
3.2.8 Acid Precipitation of Histones from Tissue Lysate.....	69
3.2.9 Statistics.....	69
<b>3.3 Results .....</b>	<b>70</b>
3.3.1 Xenoestrogen-specific activation of non-genomic signaling in the neonatal uterus	70
3.3.2 Xenoestrogen-specific modulation of EZH2 in the neonatal uterus .....	72
3.3.3 Neonatal exposure to GEN and BPA induces precocious expression of estrogen- responsive genes.....	74
3.3.4 Neonatal xenoestrogen exposure developmentally reprograms gene expression in adult myometrium in the absence of morphological reprogramming .....	79
3.3.5 Genistein but not BPA exposure increases susceptibility to uterine tumors .....	82
<b>3.4 Discussion.....</b>	<b>84</b>
<b>Chapter 4: Future Directions and Conclusion .....</b>	<b>89</b>
<b>4.1 Future Directions .....</b>	<b>90</b>

4.1.1 Future Directions to Determine Xenoestrogen-Induced DNA Methylation of Developmentally Reprogrammed Genes.....	91
4.1.2 Future Directions to Determine the Mechanism(s) of Non-Genomic Signaling and Chromatin Remodeling by Xenoestrogens in Developmentally Reprogrammed Genes	104
<b>4.2 Conclusions and Implications .....</b>	<b>108</b>
 <b>References.....</b>	 <b>138</b>
<b>Vita .....</b>	<b>168</b>

## LIST OF FIGURES

<b>Figure 1.1:</b> Hypothalamic-Pituitary-Gonadal Axis .....	<b>5</b>
<b>Figure 1.2:</b> Estrogen receptor genomic signaling .....	<b>8</b>
<b>Figure 1.3:</b> Estrogen receptor non-genomic signaling .....	<b>20</b>
<b>Figure 2.1:</b> Heat map of microarray analysis .....	<b>45-46</b>
<b>Figure 2.2:</b> qPCR of DES-reprogrammed genes in the neonatal uterus.....	<b>52</b>
<b>Figure 2.3:</b> qPCR of DES-reprogrammed genes in the adult myometrium.....	<b>54</b>
<b>Figure 2.4:</b> qPCR of DES-reprogrammed genes in tumors .....	<b>57</b>
<b>Figure 3.1:</b> Xenoestrogen-specific modulation of non-genomic PI3K/AKT signaling.....	<b>71</b>
<b>Figure 3.2:</b> Xenoestrogen-specific modulation of EZH2 and H3K27me3 .....	<b>73</b>
<b>Figure 3.3:</b> Histological and Immunohistochemical studies of neonatal Eker rat uterus .....	<b>77</b>
<b>Figure 3.4:</b> Morphological reprogramming of adult Eker rat uterus.....	<b>80</b>
<b>Figure 3.5:</b> Tumor incidence and multiplicity of Eker rats exposed neonatally to xenoestrogens .....	<b>83</b>
<b>Figure 4.1:</b> Experimental design of ultradeep bisulfite sequencing.....	<b>93</b>
<b>Figure 4.2:</b> Primer design of developmentally reprogrammed genes .....	<b>94</b>
<b>Figure 4.3:</b> Annotation CpG islands and EREs in Gria2 and Calbindin D9k.....	<b>97</b>
<b>Figure 4.4:</b> DNA methylation analysis of Gria2 in DES exposed myometrium .....	<b>99</b>
<b>Figure 4.5:</b> DNA methylation analysis of Calbindin D9k .....	<b>100</b>
<b>Figure 4.6:</b> Estrogen priming in ELT3 cells reprograms gene expression .....	<b>102</b>
<b>Figure 4.7:</b> Reprogramming of Gdf10 <i>in vitro</i> is reversed by 5-aza treatment.....	<b>103</b>
<b>Figure 4.8:</b> Proposed mechanism of xenoestrogen-induced developmental reprogramming of gene expression .....	<b>111</b>

## LIST OF TABLES

<b>Table 1.1</b> Pharmacological Properties of Xenoestrogens .....	11
<b>Table 1.2</b> Relative Binding Affinity of Xenoestrogens to ER $\alpha$ and ER $\beta$ .....	17
<b>Table 1.3</b> Gene Expression Profiles of Genes Differentially Expressed in Human and Eker Rat Uterine Leiomyoma.....	36
<b>Table 2.1</b> Estrogen-Responsive Genes Developmentally Reprogrammed by Neonatal DES Exposure .....	48
<b>Table 2.2</b> Validation of Gene Expression from Microarray Analysis of Eker Rat Uterine Tumors and Normal Myometrium .....	50
<b>Table 3.1</b> Neoatal Uterine Gene Expression in Repsonse to Xenoestrogen Exposure .....	75
<b>Table 3.2</b> Adult Myometrium Gene Expression in Response to GEN or BPA Exposure .....	75

## **List of Appendices**

<b>Appendix A:</b> Identification of Uterine Leiomyoma Genes Developmentally Reprogrammed by Neonatal Exposure to Diethylstilbestrol in Reproductive Sciences, 2008 .....	116
<b>Appendix B:</b> Methodology for ultradeep bisulfite sequencing.....	131

## **CHAPTER 1: INTRODUCTION AND BACKGROUND**

## 1.1 INTRODUCTION

Developing organisms take cues from the surrounding environment in order to prepare for the impending stimuli and to maintain homeostasis based on sensing of available energy. An important part of this sensory mechanism is the endocrine system. At critical times during its development, important feedback systems are established. As such, any aberrant stimuli or endocrine disruption can impede the normal feedback mechanism leading to improper signaling and alterations in morphology of endocrine organs, such as the reproductive tract. Endocrine disruption during development can subsequently increase the risk of neoplastic transformation of the reproductive tract and development of hormone-dependent tumors. Furthermore, endocrine disruption during developmental in individuals that are already genetically susceptible to hormone-dependent tumors are put at significantly higher risk for tumor development. However, the etiology of hormone-dependent tumors and the mechanisms by which environmental endocrine disruptors reprogram susceptibility to tumor development are still be defined. It is the purpose of this section, therefore, to address the issues surrounding developmental reprogramming of tumor risk.

## 1.2 DEVELOPMENTAL REPROGRAMMING

The theory of developmental reprogramming was originally postulated by Barker in 1989 to explain a phenomenon he and his colleagues observed in adults born in the lowest birth-weight quartile. Subsequent to their low birth-weight and low weight at 1 year these individuals had significantly higher mortality rates from cardiovascular disease and significantly higher rates of diabetes (1). Barker determined from these data that low birth weight, secondary to *in utero* growth restriction (IUGR), developmentally reprogrammed the normal response to endogenous and environmental stimuli. As a result, adult disease arose in these individuals, a concept Barker termed developmental reprogramming.

Developmental reprogramming that causes adult disease, also known as fetal origins of adult disease (FOAD), is a theory that potentiated our study of the origins of neoplasias arising in the reproductive tract. The theory that early life exposure to anthropogenic or natural chemicals could increase the risk for reproductive tract

disease was supported by a classic example in humans of developmental reprogramming by an environmental stimuli, diethylstilbestrol (DES) (2). *In utero* exposure to DES, a synthetic estrogen, led to female reproductive tract malformation and severely compromised reproductive tract function, and ultimately in some women caused the development of a rare form of vaginal adenocarcinoma (3). Several follow-up studies in DES-exposed individuals have shown the extent of reprogramming affects of DES. These reprogramming effects include an increased risk for breast cancer (4) and increased infertility in women (5), in addition to an increased incidence in testicular cancer in men (6). Importantly, recent reports have demonstrated that *in utero* exposure to DES has transgenerational effects. Daughters of women exposed to DES, called DES granddaughters, also have an increased risk of menstrual dysfunction (7) through a mechanism that is thought to be primarily epigenetic (8). These transgenerational effects have also been demonstrated in animal models. For example, female offspring (F2-F3) of F1 females neonatally exposed to DES have an increased incidence of uterine adenocarcinomas than offspring control females (9). DES is an example of an endocrine disrupting chemical (EDC). The mechanism by which DES and other EDCs function to induce developmental reprogramming is still unknown.

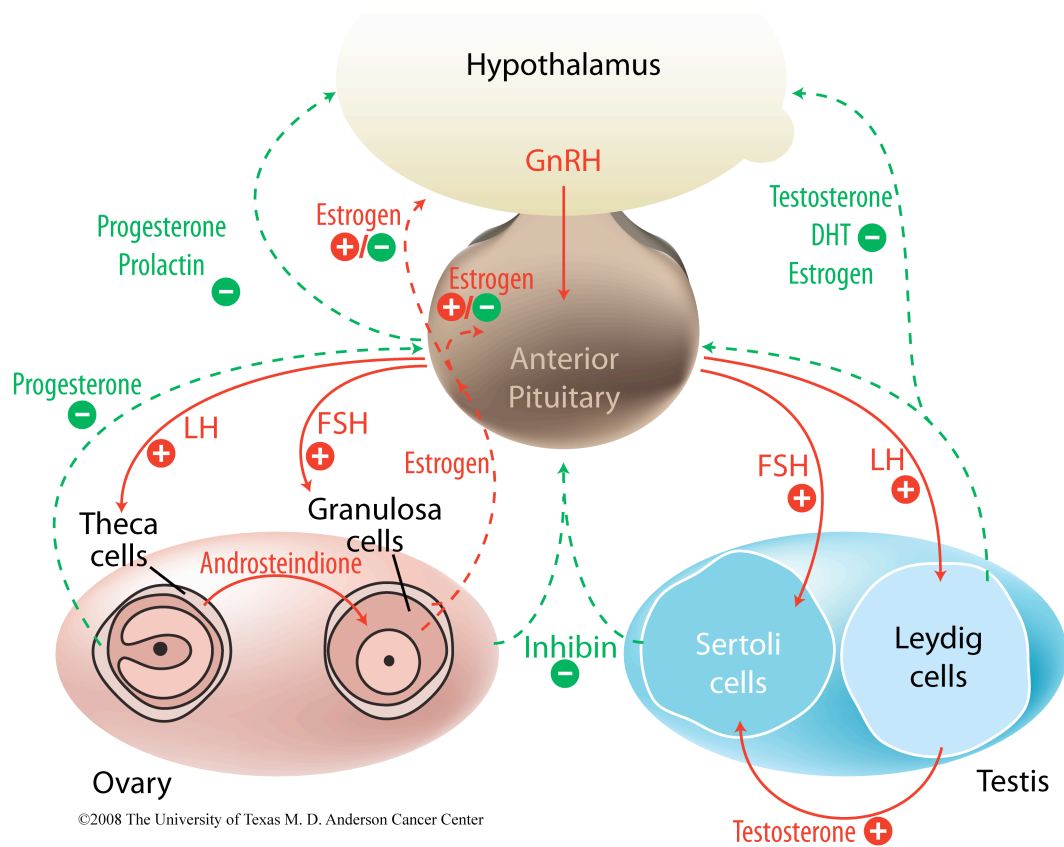
### **1.2.1 Endocrine Disruptors**

#### **1.2.1.1 SUMMARY OF THE ENDOCRINE SYSTEM**

In order to maintain metabolic homeostasis, endocrine organs send and receive signals in the form of feedback loops. The endocrine organs include the gonads, adrenals, pituitary, hypothalamus and thyroid, which function to secrete hormone(s) that act upon target tissues to produce a response through hormone binding to specific cell surface receptors. The effect produced can include both positive feedback, which is secretion of a hormone that signals the original organ to continue to release the hormone, and negative feedback, which is secretion of a hormone that signals the original organ to cease hormone release. Deregulation of these feedback loops can cause a hormone imbalance leading to metabolic disruption.

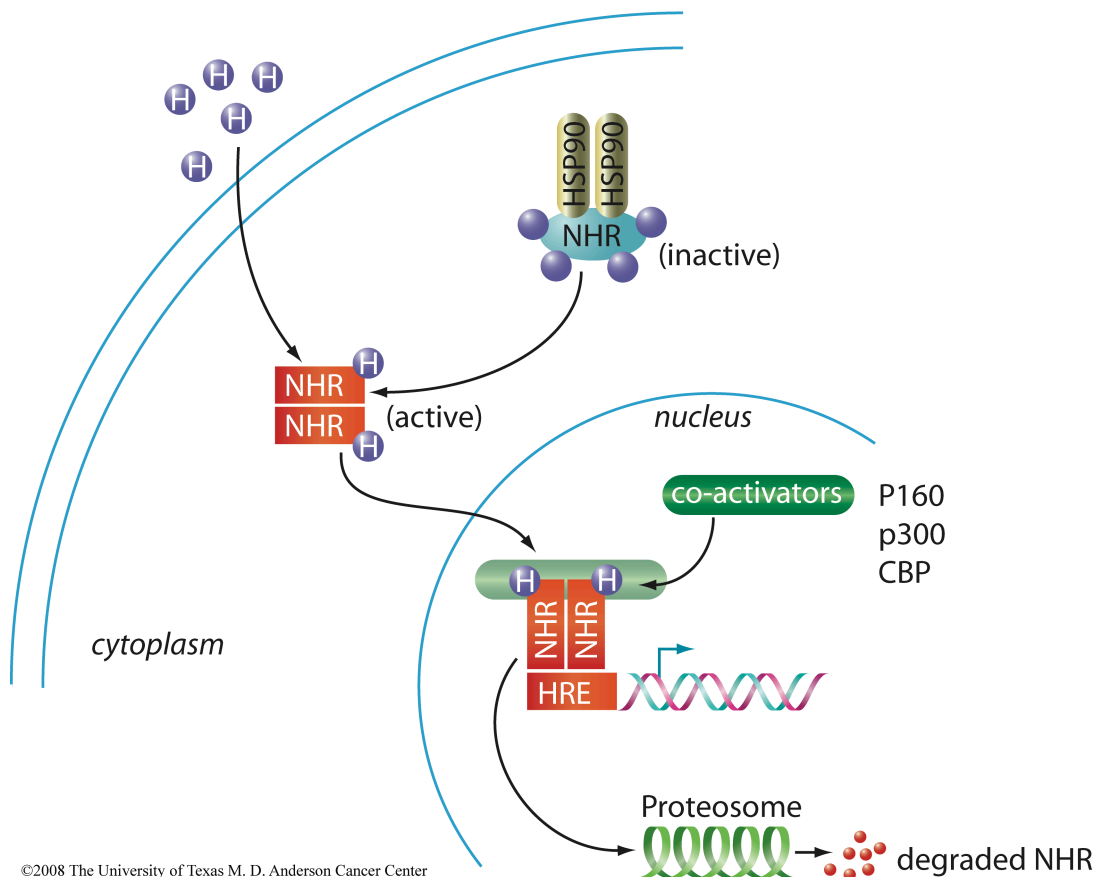
One major network of the endocrine system is the hypothalamic-pituitary-gonadal (HPG) axis. The cascade of signals from the HPG axis originates from the

hypothalamic neurons with the pulsatile release of gonadotropin-releasing hormone (GnRH). GnRH released into the blood binds GnRH receptors in the anterior pituitary, which triggers the release of luteinizing- and follicle-stimulating hormone (LH and FSH) from gonadotrope cells. LH and FSH then bind to receptors in the gonads located in granulosa and theca cells (ovary) or Leydig and Sertoli cells (testis), which culminates in their production of steroid hormones (i.e. estrogen and testosterone). In addition, other regulatory proteins such as inhibin are also produced, and these ultimately affect the response of target tissues. The cells in the gonads then send feedback signals, for example progesterone release from theca cells, which instructs the pituitary and hypothalamus to stop the release of LH, FSH and GnRH (**Figure 1.1**).



**FIGURE 1.1 – ILLUSTRATIVE REPRESENTATION OF THE MAMMALIAN HYPOTHALAMIC-PITUTARY-GONADAL (HPG) AXIS.** In the hypothalamus gonadotropin releasing hormone (GnRH) is secreted from GnRH neurons into the portal blood system. GnRH receptors in the pituitary bind GnRH, which induces the production and secretion of the gonadotropins leutinizing hormone (LH) and follicle stimulating hormone (FSH). LH and FSH then travel systemically to the ovary (females) or testis (males) to induce production of steroid hormones. In the ovary, LH binds LH receptors in the theca cells to produce progesterone and androsteindione. Androsteindione is secreted in a paracrine manner to the granulosa cells while progesterone feeds back negatively to the pituitary and hypothalamus to reduce secretion of GnRH, LH and prolactin. Prolactin secreted from the pituitary also feeds back negatively to the hypothalamus to reduce corticotrophin releasing hormone. FSH binds FSH receptors in granulosa cells to produce estradiol, which feeds back positively to the hypothalamus to increase GnRH secretion and negatively to the hypothalamus and pituitary to reduce FSH secretion. In the testis, LH binds receptors on the Leydig cells, which secrete testosterone in a paracrine manner to the Sertoli cells. Testosterone, dihydrotestosterone (DHT) and/or estradiol negatively feed back from the testis to the hypothalamus and pituitary to reduce secretion of GnRH and LH. FSH binds to receptors on the Sertoli cells stimulating them to produce inhibin B, which is also secreted from the granulosa cells, and feeds back negatively to the pituitary to reduce secretion of FSH. This illustration is representative of the HPG axis, and it should be noted that testosterone and estradiol also have other target tissues within the body for example bone and muscle.

Estrogen (e.g.  $17\beta$ -estradiol) is a key hormone that induces the transduction of signals from the female reproductive system to the pituitary and hypothalamus. In females,  $17\beta$ -estradiol (E2) is necessary for normal reproductive tract development and is produced in the human fetus at about 17 weeks of gestation (10) or around postnatal day 9 (11) in rodents. This hormone is responsible for a number of critical functions in the reproductive tract, including control of proliferation and differentiation of estrogen-responsive tissues. E2 is paramount in maintaining normal function of the ovaries, uterus and vagina, to maintain ovulation, pregnancy and menses. E2 is also a critical hormone outside of the reproductive tract, where it controls metabolic homeostasis in the bone, brain and cardiovascular system. E2 production begins when LH binds to its receptor on the theca cells in the ovary, which stimulates them to produce androstenedione. Androstenedione then traverses the basement membrane and enters the granulosa cells where, upon binding of FSH to its receptor on the granulosa cells, it is converted to  $17\beta$ -estradiol. At this time  $17\beta$ -estradiol enters the blood (12), where it targets the estrogen receptor (ER) in specific tissues to elicit its effects. The ability of estrogen to induce a response in estrogen-responsive tissues is mainly via the ER. ER is a nuclear hormone receptor that dimerizes upon its ligand binding (normally E2) and induces the expression of genes that contain an estrogen response element (ERE). Prior to binding of E2 to ER, the ER is confined to the cytosol in its inactive state, which is accomplished through the binding of ER to large protein complexes (e.g. heat shock protein 90). After E2 binds to ER, ER monomers dissociate from the inactivating protein complexes, dimerize, and translocate to the nucleus, where they bind to specific regions of DNA within genes that contain EREs in their regulatory regions (e.g. promoters and enhancers) (**Figure 1.2**). An additional mechanism that controls expression of estrogen-responsive genes is the recruitment of co-activators and co-repressors that function as initiators or repressors of transcription, which will be discussed in more detail in section 1.3.



**FIGURE 1.2 – DIAGRAM OF GENOMIC NUCLEAR HORMONE RECEPTOR SIGNALING AND TRANSCRIPTIONAL ACTIVATION.** Hormone (H) and certain EDCs can enter the cell where nuclear hormone receptors (NHR) are kept inactive by chaperone proteins including heat shock protein 90 (HSP90) until liganded by hormones (or EDCs). Liganded NHRs form dimers and translocate to the nucleus where they interact with co-activators (e.g. p160, p300 or CBP) and bind to a hormone responsive elements (HRE) in the DNA. After gene transactivation, NHRs are degraded by the proteasome or recycled back to the cytoplasm.

### **1.2.1.2 XENOESTROGEN-INDUCED DEVELOPMENTAL REPROGRAMMING**

Chemicals that disrupt the normal signaling and homeostasis of the endocrine system are termed endocrine disrupting chemicals (EDCs). EDCs are found ubiquitously in the environment and can occur naturally or as anthropogenic substances. EDCs can disrupt the endocrine system via several mechanisms, one of which is by acting as hormone mimetics. EDCs affect hormone receptor signaling by acting as agonists or as antagonists, which activate or inhibit receptor signaling, respectively. Often EDCs interfere with hormone receptor binding to endogenous hormone, and this has numerous effects, including disturbing gene expression, metabolism and hormone synthesis. One specific class of EDCs is xenoestrogens, which are chemicals that behave like or have estrogenic properties and are known to bind the ER. Given that the ER is a promiscuous nuclear hormone receptor that binds to a variety of chemical compounds, the potential for aberrant ER activation leading to perturbation of normal development, hormone homeostasis and fertility is substantial (13). The majority of xenoestrogens (including DES) act as ER agonists, mimicking the effects of endogenous E2. Alternatively, xenoestrogens can antagonize ER-DNA binding [e.g. Bisphenol A (BPA) (14)], alter co-activator/co-repressor recruitment [e.g. 4-tert-Octylphenol and BPA (15)], or increase the expression of ER co-repressors [e.g. NCOR1/SMRT (16, 17)].

The dose and route of administration of an EDC is an important factor that affects the ability of EDCs to induce developmental reprogramming effects. Several controversial issues have arisen due to the lack of continuity of dose and administration between studies assessing the effects of xenoestrogen exposure during development. The issue of dose is of importance because several xenoestrogens demonstrate a non-monotonic “U shape” dose response, including the xenoestrogens evaluated in this work (18). Because of the non-linear dose response seen between high and low doses of xenoestrogens, the extrapolation of low or physiologically relevant doses from high or pharmacological doses is not generally prudent. In addition, the ability of neonatal animals to metabolize xenoestrogens has also been a confounding factor due to the neonate’s immature Phase II metabolism (glucuronodation) as compared to adult rodents (19-21). This disparity has created

another variable that introduces variation between studies, making comparisons and extrapolations difficult.

For example, the isoflavone, genistin (GIN), which is the primary component of soy-formula, is present in the lumen of the intestinal tract as a glycoside/glucoside. This glycoside undergoes hydrolysis in the small intestine via beta-glycosidase, which is an enzyme that is associated with lower bowel microflora and is requisite for absorption of glycosylated chemicals. After absorption, the deglycosylated (aglycone), now the EDC genistein (GEN), is metabolized by the Phase II enzymes uridine-5'-diphosphate glucuronosyl-transferases, and sulfotransferases, and is excreted in the bile or urine. Conjugates in the bile are hydrolyzed by intestinal bacteria and excreted in the feces, or they undergo further metabolism through re-absorption into enterohepatic circulation, or they are degraded. The major products resulting from metabolism found in the hepatic portal vein are glucuronides (22).

Since the aglycone form, GEN, is the “estrogenic” or active form, it is important to know the amount of genistein aglycone that is present in plasma, because this is a measure of the body’s ability to metabolize aglycone via glucuronodation (i.e. its ability to inactivate and excrete GEN). Perinatal rodents have reduced glucuronidation, and this is exemplified in the half-life of genistein aglycone, which is higher in neonatal animals as compared to adults (12-19 hours vs. 2-4 hours, respectively) (23, 24). The EDC BPA is processed in a similar fashion, and fetal and neonatal rats do not efficiently metabolize BPA because their enzyme systems are not fully matured. For example, an oral dose of 10 mg/kg in post-natal day (PND) 4 rats results in 100-1000 times higher concentrations of BPA than the same dose in adult rats. However, at doses of <1mg/kg neonatal rats are able to efficiently metabolize BPA to the glucuronidated form. Importantly, oral vs. subcutaneous (s.c.) routes at low doses (35 or 395 ug/kg) in neonates does not result in significant differences in plasma concentrations (25). Therefore, the factors of dose, route of administration, and age of the animal were all taken into consideration when designing the following experiments. In addition, extrapolations from the data presented herein were carefully analyzed in the context of the parameters discussed above. The doses, routes and plasma concentrations of the xenoestrogens used in this work are listed in **Table 1.1** and are shown in relationship to the physiologically relevant exposure level in the age-appropriate human population.

**Table 1.1**

Pharmacological Properties of Xenoestrogens in Neonatal Rodents vs Human Exposure

	DES		GEN		BPA	
	dose	plasma (uM)	dose	plasma (uM)	dose	plasma (uM)
Dose (rodent)	1mg/kg <sup>d</sup> (sc)	5 <sup>a</sup>	50 mg/kg <sup>e</sup> (sc)	6.8 <sup>b</sup>	50 mg/kg <sup>f</sup> (sc)	3-30 <sup>c</sup>
Relevant Human Exposure	1.5-150 mg/d <sup>j</sup>		1-8 mg/kg (po) <sup>g</sup>	1-6 <sup>h</sup>	2.42 µg/kg (po) <sup>i</sup>	.001-.010 <sup>g</sup>
Half-life (t <sub>1/2</sub> )	1.9 hrs <sup>k</sup>		12-19 hrs		>6.7 hrs	

<sup>a</sup> dose estimated from Miller et al. *J Pharm Exp Therap* 1981<sup>b</sup> dose confirmed by Doerge et al. *Cancer Letters* 2002<sup>c</sup> dose estimated from Taylor et al. *Reproductive Toxicology* 2008<sup>d</sup> estrogenic dose, Cook et al. *PNAS* 2007<sup>e</sup> estrogenic dose, Jefferson et al. *Environmental Health Perspectives* 2009<sup>f</sup> estrogenic dose, unpublished results<sup>g</sup> US Department of Health and National Toxicology Program Brief on BPA, 2008<sup>h</sup> Food Standards Agency COT Working Group on Phytoestrogens Draft Report, 2002<sup>i</sup> FDA Report on Bisphenol A, 2008<sup>j</sup> Marselos et al. *Eur J Cancer*, 1992<sup>k</sup> Ben et al. *Analytical Biochemistry*, 2005

TABLE 1.1

#### **1.2.1.2.1 DES AND DEVELOPMENTAL REPROGRAMMING**

The potent synthetic estrogen DES is a classical example of a xenoestrogen that acts as an EDC. DES, which was used from 1940-1970 as a pharmaceutical agent, was prescribed to pregnant women to prevent miscarriages and premature delivery. Approximately 2-8 million Americans were exposed *in utero* to DES before the FDA banned its use in 1971 (26). The ban of DES was predicated upon multiple studies and case reports documenting exposure-related development of a rare vaginal cancer, vaginal clear cell carcinoma in early adult post-pubertal girls, in addition to reproductive tract malformations, infertility, preeclampsia and the benign uterine neoplasias, uterine leiomyomas (27-31). Similarly, males exposed *in utero* to DES did not escape the developmental reprogramming effects, as evidenced by reports documenting testicular hyperplasia and hypospadias, epididymal cysts and impaired spermatogenesis (32, 33). In subsequent follow-up studies of women exposed *in utero* to DES, a significant correlation was found between exposure and an increased risk of breast cancer (34). These studies indicate that DES is a transplacental carcinogen that may act via developmental reprogramming of the reproductive tract, demonstrating the sensitivity of differentiating tissues to endocrine disruptors.

The developmental reprogramming effects of DES exposure have also been well-documented in numerous animal studies (33). Specifically, studies in CD-1 mice demonstrate that postnatal exposure to DES (PND 1-5) induces the formation of uterine adenocarcinomas, and to a lesser extent, uterine leiomyomas (35, 36). Characterization of the developmental reprogramming effects of postnatal DES exposure in the reproductive tract revealed malformations in the ovary and oviduct (37, 38). In addition, alterations in the epithelium of the uterus and vagina, increased vaginal epithelial cornification (35, 39) and alterations in uterine gland formation (40) have also been identified. However, *in utero* exposure of mice to DES, in contrast to the postnatal exposure data presented above, does not result in significant development of vaginal adenosis. This is in comparison to mice exposed to DES on PND 1-5, which gave rise to a 75% incidence of vaginal adenosis in adulthood (41). Similarly, exposure to DES postnatally and postweaning induced significant

developmental effects as compared to *in utero* exposure (42). These data further substantiate the postnatal period as the most susceptible window for DES-induced developmental reprogramming of the reproductive tract in rodents. The main reason postulated for the differences in the window of susceptibility to DES exposure between humans and rodents can be attributed to the periods of differentiation and development of the uterus. While the majority of human uterine development is completed between 19-38 weeks of gestation, rodent uterine development is completed between PNDs 3-19 (43).

#### **1.2.1.2.2 GENISTEIN AND DEVELOPMENTAL REPROGRAMMING**

While the lingering effects of DES remain as evidence of the millions of individuals exposed to DES *in utero*, the lessons learned from DES exposure have led to the investigation of the effects of other natural and anthropogenic xenoestrogens. The phytoestrogen, GEN, is one such xenoestrogen that has been studied extensively, because it is found in soy infant formulas and multiple other food products that are consumed by infants. The concern regarding infants consuming soy-based formula is the high amounts of soy isolates, such as GIN, that infants are exposed to during critical developmental windows. In comparison to adults who consume soy-based foods, infants consuming soy formula are exposed to approximately 10 times the amount of genistein (6-9 mg/kg/d vs. 1 mg/kg/d) (44). To date, 3 epidemiological studies of human infants consuming soy-formula have demonstrated adverse affects of exposure. These reports have correlated consumption of soy-formula, as compared to breast- or cow-milk, with increased breast tissue at 2 years of age (45), with a higher maturation index of cells from the vaginal wall (46), as well as with increased incidence of uterine leiomyoma (47). Importantly, GEN has also been demonstrated at environmentally relevant doses (1.4-5.4  $\mu$ M in plasma) (23, 48, 49) to induce developmental reprogramming of the female reproductive tract and mammary glands in numerous animals studies (20). While animal studies show that genistein does cross the placental barrier (50), the more relevant neonatal exposure to GEN in rodents leads to both carcinogenic effects, including uterine adenocarcinomas (51, 52), as well as non-carcinogenic effects. These effects include infertility, altered estrous cyclicity, accelerated vaginal opening, early reproductive senescence,

multioocyte follicles and multiple histopathological alterations of the reproductive tract and mammary glands (52-58).

As with DES exposure, the timing of GEN exposure in animals alters the differentiation and susceptibility of exposed tissues, such as the mammary glands. Unlike the uterus, however, the differentiation of the mammary gland is similar between humans and rodents with the majority of development taking place before birth. *In utero* versus neonatal GEN exposure, however, differentially alters the susceptibility to carcinogen-induced mammary carcinogenesis in rodent studies. If GEN exposure occurs *in utero*, carcinogen-induced mammary cancer is increased, whereas there is a chemopreventative effect on mammary cancer if exposure occurs neonatally (59-61). These studies demonstrate that changes in GEN-induced differentiation of the mammary gland correlate with the induction or prevention of mammary tumors. The ability of GEN to alter tissue differentiation, is a component this xenoestrogen utilizes to induce developmental reprogramming. These studies illustrate the different developmental windows of susceptibility to carcinogenesis from xenoestrogen exposure.

#### **1.2.1.2.3 BPA AND DEVELOPMENTAL REPROGRAMMING**

Another endocrine disruptor, bisphenol A (BPA), was originally synthesized at the same time as DES and was to be used as a synthetic estrogen (62, 63). However, it later became used prolifically as a cross-linking chemical in production of epoxy resins and polycarbonate plastics. Due to its chemical instability, BPA easily leaches from plastics after heating and contaminates food and beverages. BPA is also used in can liners and dental sealants, which confers another avenue of human exposure. Recently, correlation of urinary BPA concentration in human studies has been positively associated with heart disease and diabetes (64, 65). Furthermore, several studies, including a study from the CDC, demonstrated that BPA can be found in nanomolar concentrations in urine, serum and breast milk (66-68). Importantly, human studies of fluids from the maternal-fetal-placental unit have also identified concentrations of BPA (0.2 – 104 ng/mL (6 nM in cord blood)) that are within the range of those that have shown toxic effects in offspring from animal studies. This indicates that BPA can cross the placental barrier (69).

In several animal studies, *in utero* and neonatal BPA exposure results in reproductive tract alterations in both males and females. *In utero* exposure alters oogenesis (70), advances puberty, (69) and changes endometrial gland proliferation, uterine epithelium and vaginal morphology (71-73). Similar to *in utero* exposure, neonatal exposure to BPA alters oogenesis (74), however, neonatal BPA exposure also induces alterations in estrous cyclicity (75) and steroid hormone-response in the uterine stroma (16) and persistent vaginal cornification (76). Importantly, neonatal BPA exposure promotes neoplastic transformations, including cystic endometrial hyperplasia (64, 77) and age-related prostatic intraepithelial neoplasia (78). With the results of these rodent studies showing the deleterious effects of both *in utero* and neonatal exposure, in combination with the wide range of concentrations of BPA found in humans, the potential for developmental reprogramming of the human reproductive tract and promotion of neoplastic transformation from BPA exposure is apparent.

### **1.3 MECHANISMS OF DEVELOPMENTAL REPROGRAMMING**

While the exact mechanism by which xenoestrogens induce developmental reprogramming remains elusive, several theories have been explored. The main mechanism of action through which xenoestrogens have been shown to affect cellular function is via activation or repression of nuclear hormone receptors (NHR). While hydrophilic xenoestrogens initiate rapid signaling via membrane-bound NHRs, other lipophilic xenoestrogens passively enter the cell to activate NHRs and regulate expression of estrogen-responsive genes (79). NHRs are activated by steroid hormone ligands, or by chemicals that behave like steroid hormones (e.g. xenoestrogens). Hormone ligands induce a conformational change in the protein structure of the NHRs that allows dimerization and translocation to the nucleus, where they act as transcription factors that result in expression or repression of genes. Accessory proteins such as co-activators and co-repressors also often manipulate the function of NHRs by recruiting other proteins or by tethering the complex to the DNA. While xenoestrogens predominately act on Type I NHRs (e.g. androgen receptor (AR), progesterone receptor (PR) and estrogen receptor (ER)), other EDCs bind to Type II or Type III NHRs, which are normally liganded by non-steroid hormones or act as orphan receptors, respectively.

### 1.3.1 Xenoestrogen-Induced Genomic Mechanisms of Endocrine Disruption

Xenoestrogens transactivate estrogen-responsive genes through binding to ER to elicit a genomic response. ER is a Type 1 NHR that is expressed as either ER $\alpha$  or ER $\beta$ , which form either homo- or heterodimers with one another (80). Both isoforms contain similar conserved domains, which include an N-terminal transactivation domain, a zinc finger DNA binding site and a C-terminal ligand-binding domain. However, the ER isoforms can act antagonistically towards each other. For example, ER $\beta$  can promote apoptosis and inhibit the proliferation induced by ER $\alpha$  (81). Once ER is liganded, dimers are formed that translocate to the nucleus where they bind specific regions in the DNA, classically termed estrogen response elements (ERE). In order to activate transcription of ERE-containing genes, other coregulatory proteins are required, such as steroid receptor coactivator 1 (SRC-1) (82).

Xenoestrogens can act as both agonists and antagonists of ER activity. DES, for example, can bind ER $\alpha$  and induce transcriptional activation of *HOX* genes, such as *Hoxa9* and *Hoxa10*, which contain EREs in their promoters (83). However, with other xenoestrogens, such as GEN, both agonism and antagonism of ER can occur. *In vitro*, for GEN to act as an ER agonist, low-doses (<9  $\mu$ M) are required, and they induce cell growth. At high-doses (9-100  $\mu$ M) GEN inhibits activation of downstream signaling via receptor tyrosine kinases (RTKs) (84, 85) and subsequently cell growth. BPA can also act as an ER antagonist by inhibiting ER binding to DNA (14), in addition to preferentially recruiting ER co-repressors to estrogen-responsive genes. Another mechanism by which xenoestrogens can alter genomic responses is via differential binding to ER $\alpha$  versus ER $\beta$ . As indicated previously, ER $\alpha$  activity can be inhibited by liganded-ER $\beta$ . As shown in **Table 1.2**, xenoestrogens have variable affinity for the different isoforms of ER. Specifically, *in vitro* studies indicate that GEN has a stronger binding affinity for ER $\beta$  versus ER $\alpha$  (15, 86). Due to the tissue-specific expression differences in ER $\alpha$  versus ER $\beta$  (e.g. uterus expresses mainly ER $\alpha$  and the prostate expresses mainly ER $\beta$ ), the binding affinity may account for the differential effects of GEN, BPA and DES *in vivo*. Animal studies have also demonstrated xenoestrogen-specific effects on the reproductive tract, lending further support to this theory (87).

Chemical	Relative Binding Affinity					
	ER $\alpha$		ER $\beta$		Ratio ER $\beta$ /ER $\alpha$	
	RBA <sup>a</sup>	RBA <sup>b</sup>	RBA <sup>a</sup>	RBA <sup>b</sup>	ratio <sup>a</sup>	ratio <sup>b</sup>
<b>17<math>\beta</math>-Estradiol</b>	100	N/A	100	N/A	1	N/A
<b>Diethylstilbestrol</b>	53	100	150	100	2.8	1
<b>Genistein</b>	0.7	1.4	15	88	21.4	62.9
<b>Bisphenol A</b>	0.073	0.2	0.75	1.2	10.3	6

<sup>a</sup>Routledge et al. 2000

<sup>b</sup>Mueller et al. 2003

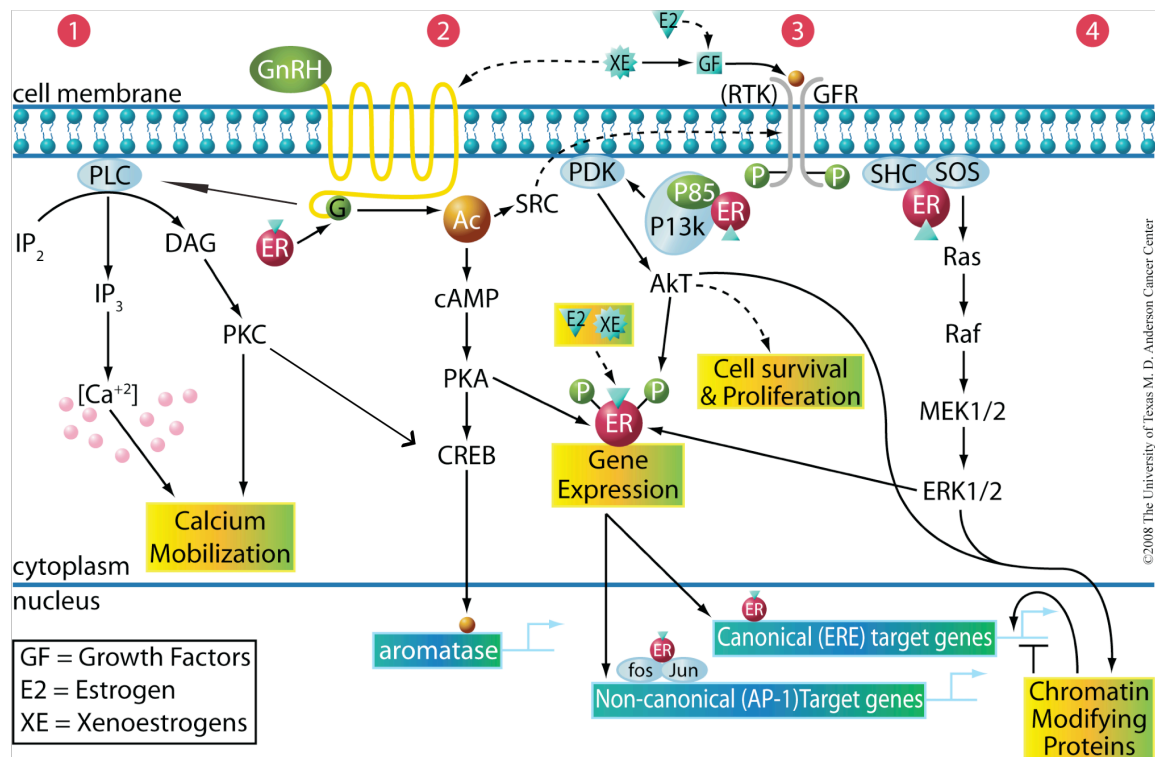
TABLE 1.2

### 1.3.2 Xenoestrogen-Induced Non-Genomic Mechanisms of Endocrine Disruption

In addition to genomic activation, xenoestrogens can also activate NHRs via non-genomic signaling, which takes place in the cytoplasm and is independent of NHR-DNA binding. As compared to genomic signaling, which occurs slowly (hours → days), non-genomic signaling is rapid (seconds → minutes). The ability of hormones to induce non-genomic activation was demonstrated originally by Losel and Wehling (88) utilizing spermatozoa, which do not contain a 'normal' nucleus and thus lack genomic signaling. Evidence of xenoestrogen-induced non-genomic signaling came from the demonstration that xenoestrogens still have an effect on nucleated cells treated with inhibitors of protein synthesis, which blocks the downstream effectors of genomic signaling. Furthermore, xenoestrogens that cannot enter the cell still elicit signaling events, mimicked by BSA bound E2, and this must also be a genomic signaling-independent mechanism. Thus, the ability of xenoestrogens to interfere with normal hormone signaling via non-genomic signaling could also give rise to developmental reprogramming.

Multiple non-genomic signaling pathways can be induced by estrogen (**Figure 1.3**). Normally, E2 induces  $\text{Ca}^{++}$  mobilization (89), elevates  $\text{Ca}^{++}$  concentrations, (90) activates growth factor receptors and G-protein coupled receptors (GPCR) (Thomas 2005) (91) as well as protein kinases (92, 93) via ligand bound ER and non-classical ERs. However, xenoestrogens, including DES, GEN and BPA, can also give rise to non-genomic signaling events. In the reproductive tract, E2, as well as xenoestrogens, induce fluctuations in  $\text{Ca}^{++}$ -dependent signaling, leading to changes in apoptosis, neuronal transmission, muscle contraction and gene expression. Downstream signaling events of  $\text{Ca}^{++}$  mobilization subsequently activate kinases in an ER-dependent manner, such as mitogen-activated protein kinases (MAPK) and protein kinase C (PKC) (89, 93), which are responsible for regulating mitogenic, stress and inflammatory signals. However, both E2 and xenoestrogens can activate multiple kinases (e.g. Ras-Raf-MEK-ERK/MAPK) *in vitro* in an ER-dependent manner that is independent of  $\text{Ca}^{++}$  signaling (94-96). Additionally, E2 and xenoestrogens have been shown to activate certain GPCRs (97), though this is separate from the membrane-bound ER (mER $\alpha$ ). However, the ability of GPCRs to mediate estrogen signaling is controversial (98, 99). Thomas *et al.* (100, 101) demonstrated in ER-null breast cancer

cells (SKBr3) that GPR30 could bind not only E2, but also the xenoestrogens, ortho,para-dichlorodiphenyldichloroethylene (o,p'-DDE), GEN and BPA, which induced the activation of adenylate cyclase. The second messenger produced by adenylate cyclase and ATP, adenosine 3', 5'-monophosphate (cAMP), binds protein kinase A (PKA), which allows PKA (R subunit) to phosphorylate cAMP response-element-binding protein (CREB) (102, 103). As a transcription factor, CREB can aid in transcription of estrogen-responsive genes including aromatase and c-fos/c-jun (104). Recently, Bouskine *et al.* (105) reported that in human seminoma cells, BPA, but not E2 or DES, was able to induce proliferation by activation of PKA and PKG through binding to a GPC non-classical ER. However, non-genomic activation was independent of classical ER, since the ER antagonist, ICI 182780, could not abrogate the signaling. E2 bound to BSA (which cannot enter the cell), however, was able to produce the same effect as BPA, i.e. phosphorylation of CREB, indicating the xenoestrogen-specific effects on non-genomic signaling.



**Figure 1.3 – Mechanisms of Estrogen and Xenoestrogen-Induced Non-Genomic Signaling.** 1) Calcium mobilization, as the result of non-genomic signaling through protein kinase C (PKC) via activation of GPCRs by liganded ER, is one of the consequences of estrogen or xenoestrogen exposure. 2) Activation of GPCRs by estrogen or xenoestrogens also induces non-genomic signaling and expression of estrogen-responsive (canonical and non-canonical) genes via protein kinase A (PKA) activation of ER. Estrogen-induced GPCR activation also signals via CREB to induce aromatase expression. 3) In addition to GFs, estrogens and xenoestrogens can promote cell survival, proliferation and estrogen-responsive gene expression via activation of the PI3K/Akt and MAPK pathways. Activation of GFRs can also induce PI3K and MAPK signaling, which can both phosphorylate the ER. Alternatively, liganded-ER can bind directly to the p85 subunit of PI3K to activate Akt or bind Shc to activate the ERK1/2, which can also phosphorylate ER. 4) Chromatin modifying proteins can also be posttranslationally modified by estrogen-activated AKT, which alters histone marks on chromatin giving rise to changes in expression of estrogen-responsive genes.

While both isoforms of ER have been shown to reside in the cytosol, nucleus and mitochondrial membranes (102, 106, 107), putative non-classical membrane-bound ERs are still debated because ER does not contain a transmembrane domain. Razandi *et al.* (102), however, was able to demonstrate that ER-negative Chinese hamster ovary (CHO) cells transfected with ER produced ER in both the cell membrane and nucleus. ER was also found to associate with several membrane signaling proteins including Src, Shc and caveolin-1. Overexpression of caveolin-1 was demonstrated to induce translocation of nuclear ER to the cytoplasm, resulting in ER-dependent kinase activation (108, 109). Despite the research being done on membrane-bound ER, the exact mechanism, be it via association with other membrane-bound proteins or posttranslational modification, by which ER associates with the membrane remains enigmatic.

Importantly, growth factors, E2 or xenoestrogen-induced kinase activation results in ER $\alpha$  phosphorylation at Ser118 (by, for example, MAPK and PKC) and Ser167 (e.g. by Akt) (110), which can recruit co-activators of ER, leading to changes in estrogen-responsive gene expression (111-115). Growth factors such as EGF and IGF-1, however, can also lead to ER $\alpha$  phosphorylation via kinase activation by enzymes such as MAPK, independently of E2 binding (116). In addition, ER can bind directly to p85 (117), a subunit of phosphoinositol-3-kinase (PI3K), as well as to Erb2/Her2 receptor tyrosine kinase (114), in an E2-dependent manner, which leads to both activation and phosphorylation of Akt. The phosphorylation of Akt has multiple downstream effects, which ultimately results in cell survival. Xenoestrogens, including DES (118), GEN (at low doses; < 50 $\mu$ M) (119) and BPA (93) can also activate the PI3K pathway *in vitro*. An important recent finding of the E2/xenoestrogen-mediated activation of Akt is the downstream regulation of a histone modification enzyme, enhancer of zeste homolog 2 (EZH2), which is a histone methyltransferase (HMT). The phosphorylation of EZH2 by Akt was identified *in vitro* as a downstream signaling event by insulin-like growth factor (IGF), which decreased the HMTase activity and association of EZH2 with chromatin (120). The resulting event was the decreased trimethylation of histone H3 at lysine 27 and increased gene expression. Subsequently, it was found both *in vitro* and *in vivo* that DES can also result in increased pEZH2 in an ER-dependent mechanism. Furthermore, this increase in pEZH2 was abrogated in

mice lacking ER $\alpha$  (ERKO mice). These data demonstrate another mechanism of xenoestrogen-induced developmental reprogramming via non-genomic signaling as well as histone modification.

### **1.3.3 Epigenetic Developmental Reprogramming**

Early developmental biologists and geneticists recognized that DNA sequence is insufficient to give rise to the tremendous variation in phenotype found in higher organisms (121) (8). Conrad Waddington was the first to use the term “epigenetics” to describe the means by which differentiation makes traits reproducible due to the establishment and maintenance of gene expression profiles through a process called “canalization” (122) (123). The word epigenetics is now used to describe the molecular mechanisms that make gene expression patterns and subsequent traits heritable in a manner independent of DNA sequence variations. Fetal development is a powerful illustration of the importance of epigenetic regulation in which the DNA sequence from a single cell generates all cell types and tissues of the body (121).

While epigenetic structures found in various cell types must be stable and heritable to produce distinctive traits found in tissues of the body, the epigenome is also dynamic, which allows gene expression profiles to change in response to environmental stimuli. Importantly, such responses are appropriate and normal to enable organisms to protect themselves from environmental stressors or to adapt to new environments. However, inappropriate stimulation of these same pathways during development, when the fetus is particularly sensitive to environmental factors, could result in aberrant changes in epigenome (124). Monozygotic twins are a classic example of the influence of the environment on the epigenome. Despite having identical DNA sequences, disease incidence is variable among identical twins, suggesting that environmental factors are promoting the observed differences (125).

Because environmental stimuli clearly affect epigenetic structures and subsequent disease susceptibility, it is critical to further investigate the mechanisms by which epigenetic structures are perturbed by environmental factors. Unfortunately, little is known about how environmental chemicals, such as endocrine disruptors (ECDs), alter the epigenome and contribute to the etiology of diseases such as obesity, diabetes, infertility, and cancer. The purpose of this section is to review the

mechanisms of epigenetic gene regulation, specifically histone modification and DNA methylation, as well as, to summarize what is known about the effect of EDCs on the epigenome.

### **1.3.3.1 DNA METHYLATION**

DNA methylation was the first epigenetic modification to be identified and is the best characterized epigenetic structure (126) (127). Methylation occurs by transfer of a methyl group to the cytosine found in a cytosine guanine dinucleotide (CpG) by enzymes termed DNA methyltransferases (DNMTs). The majority of CpGs are found within regions of the genome called CpG islands (CGIs). CGIs are unique regions of DNA that have an high (>50%) GC content (128) (129). CGIs are generally found in the 5' promoter region of genes and often contain structures critical to the regulation of the associated gene. In fact, 50-70% of all genes possess a CGI, which is generally unmethylated (130) (131) (132) (133).

As mentioned, DNMTs are responsible for the transfer of a methyl group to cytosine. There are four isoforms of this enzyme found in mammals, DNMT1, DNMT2, DNMT3a, and DNMT3b. With the exception of DNMT2, which only weakly methylates DNA (129) and is thought to methylate other cellular targets (134), DNMTs are critical for normal development as demonstrated in DNMT knockout mouse models, which have significant genetic defects and die during development (135) (136) (137) (138) (139) (140). The isoforms of DNMTs each have unique functions. DNMT3a and 3b, also called *de novo* DNMTs, are responsible for the establishment of DNA methylation patterns during fetal development (141). Unlike *de novo* DNMTs, DNMT1 is responsible for the propagation of DNA methylation patterns during cell division (133).

The effect of DNA methylation on gene expression was discovered using an inhibitor of DNMTs called 5-deoxy-azacytidine (5azaC), which is not only a DNMT antagonist but also cannot be methylated when incorporated into DNA. Using this antagonist, genes formerly silenced during development, such as those found on the X chromosome, could subsequently be expressed (142) (143) (144). These groundbreaking studies revealed that DNA methylation participates in silencing of gene expression. It is now appreciated that DNA methylation is not only a critical part

of fetal development, but also a critical mechanism in multiple disease states. For example, aberrant tumor suppressor gene silencing via DNA methylation allows for unrestricted division in cancer cells (145) (146) (133).

DNA methylation silences gene expression by creating docking sites for proteins called methyl-CpG-binding proteins (MDBs) (147). MDBs bind to methylated DNA and recruit histone modifying enzymes, including histone methyltransferases (HMTs) and histone deacetylases (HDACs), that promote the formation of a closed chromatin conformation, or heterochromatin. The closed chromatin conformation prevents binding of transcription factors and other coactivator complexes to gene promoters and as such prevents gene expression (148) (149) (150) (151).

#### **1.3.3.2 HISTONE MODIFICATION**

DNA is bound to scaffold proteins called histones, forming a higher order structure referred to as chromatin. Chromatin is made up of an octamer of four histone proteins, H2A, H2B, H3, and H4. In addition, Histone H1 is a linker protein found between nucleosomes. Chromatin structure is dictated by posttranslational modifications (PTMs) that occur on DNA and histone proteins. The N-terminal region of histone proteins, or histone “tails”, are subject to a number of PTMs, including phosphorylation, ubiquitination, sumoylation, acetylation, and methylation. These PTMs correspond to either promotion or inhibition of gene expression, in general. The combination of PTMs found on histones in a given region of the genome generate binding sites for domains of other regulatory proteins, such as chromo- and bromodomain containing proteins, that bind to these chromatin “reading” domains and promote binding of “effectors”. The effect that binding of the “effectors” to PTMs on histones has on chromatin ultimately governs gene expression (152) (153) (154).

HMT enzymes catalyze histone methylation. Unlike DNA methylation, site-specific histone methylation can cause induction or repression of transcription (152). Two types of HMT enzymes known to exist, which are classified by the amino acid that they methylate on histone proteins, are histone lysine methyltransferases (HKMTs) and protein arginine methyltransferases (PRMTs). In contrast to DNA methylation, histone methylation is reversible and is known to fluctuate in response to cellular stimuli (155). For example, histone lysine methylation is removed by histone

demethylases, including lysine-specific demethylase 1 (LSD1) and Jumonji domain-containing hydroxylases (JMJDs) (156) (157) (158). Another mechanism that allows for rapid fluctuation in histone methylation, as mentioned previously, is the ability of HMTs to be post-translationally modified by upstream signal transduction pathways. In addition to the ability of AKT to induce phosphorylation of EZH2, other kinases have also been demonstrated to phosphorylate HMTs. Specifically, coactivator-associated arginine methyltransferase 1 (CARM1) phosphorylation (by an unknown kinase) results in a reduction of its substrate affinity and enzymatic activity as demonstrated by Higashimoto *et al.* (159) and Feng *et al.* (160). While several groups have postulated that phosphorylation of HMTs alters protein conformation leading to reduced enzyme activity or its interaction with its different co-factors (i.e. SETDB1 phosphorylation is required for CHD7 recruitment (161)), Feng and colleagues have illustrated the precise effect phosphorylation has on CARM1 structure and enzyme activity. Solving the crystal structure of CARM1 allowed them to show that phosphorylation at Ser 217 disrupted the hydrogen bond of the hydroxyl group with the carbonyl oxygen atom of Tyr 154. Normally, this hydrogen bond locks in S-adenosylmethionine (SAM) and provides enzyme activity for CARM1, however disruption of this bond by phosphorylation inhibits SAM binding. Furthermore, Ser 217 phosphorylation also increased cytoplasmic localization. Besides phosphorylation, other PTMs such as GlcNAcylation, which is catalyzed by an O-GlcNAc transferase sensitive to serum glucose, can also alter activity of HMTs. For example the GlcNAcylation of HKMT myeloid/lymphoid or mixed-lineage leukemia 5 (MLL5), allows for its HMT activity to catalyze the mon- and dimethylation of H3K4 (162). Despite being reversible, histone methylation patterns are heritable and also appear to be, at least in some cases, precursors for other epigenetic modifications such as DNA methylation (163) (164) (165).

### **1.3.4 Xenoestrogen-Mediated Epigenetic Reprogramming**

The mechanism(s) by which early life exposure to endocrine disrupting chemicals such as xenoestrogens developmentally reprograms reproductive tract tissue are largely unknown. However, it is now appreciated that modulation of epigenetic structures to permanently alter gene expression is a major mechanism of

EDC-induced developmental programming. This is thought to be due to the fact that the fetus has a very dynamic epigenome during development and continuously subject to change prior to reaching phases of terminal differentiation. The epigenome is very sensitive to environmental stimuli, including exposure to heavy metals EDCs, which can disrupt epigenetic structures by interrupting the normal sequence of structural changes occurring during development. The timing of exposure is also critical to the outcome of these exposures. Known windows of development sensitive to reprogramming include gamete maturation (oogenesis and spermatogenesis), prior to zygote implantation when *de novo* methylation occurs, and during lineage-specific differentiation (166) (141) (167). Developmental reprogramming to gametes may cause abnormal epigenetic modifications and subsequent changes in gene expression and disease incidence to become inherited to offspring. This phenomenon is called transgenerational epigenetic effects.

Early life exposure to EDCs has been linked to changes in DNA methylation patterns in adult tissues, which corresponds to altered gene expression patterns. For example, neonatal exposure to DES reprogrammed the expression of tumor-associated genes such as *cyclin D1*, *lactoferrin*, and *transforming growth factor beta* in the uterus CD-1 mice (36) (168) (169). Li et al. (170) (171) demonstrated that neonatal DES exposure induces hypomethylation and persistent expression of the *c-fos* and *lactoferrin* genes. Similarly, neonatal exposure to BPA and DES caused hypermethylation in the *Hoxa10* gene, correlating with reduced expression in adult uterine tissue (172) (16) (173). Tang and colleagues (REF) also showed that reduced methylation of the nucleosomal binding protein 1 (*Nsbp1*) promoter following neonatal DES or genistein exposure was associated with elevated gene expression and increased tumor incidence. Remarkably, changes in DNA methylation imparted by DES-exposure are insufficient to induce uterine tumors, as ovariectomized CD-1 mice neonatally exposed to DES fail to develop uterine tumors. These data bring to light the critical role of endogenous hormone to the increased uterine tumor formation, suggesting that it is the interaction between the imprint caused by DES and the natural hormonal milieu that modulates tumor risk (35) (169) (174).

Like the female reproductive tract, the male reproductive tract is also susceptible to EDC-induced developmental reprogramming. Ho and colleagues (175) revealed that BPA exposure promoted hypomethylation of the phosphodiesterase

type 4 variant 4 (PDE4D4) gene. Importantly, increased expression and activity of the PDE4D4 of this gene produced elevated levels of camp, which was associated with increased formation of intraepithelial neoplasia (PIN) lesions in adult animals. Interestingly, the reproductive tract is not the only organ system reprogrammed by exposure to EDC's during development. Various hormone-responsive tissues are subject to EDC-induced developmental reprogramming. One of the most fascinating and poorly understood targets of these chemicals is the brain. Recent studies have demonstrated that fetal or neonatal exposure to EDCs affects morphology in specific regions of the brain such as the hypothalamus, distribution of brain hormone receptors, and alters adult behaviors (176), (177). Waalkes and colleagues observed that inorganic arsenic gave rise to hypomethylation and elevated expression of ER in liver of mice exposed *in utero*. These data reveal that early life exposure to EDCs may affect several organ systems to give rise to numerous physiological changes.

### **1.3.5 Xenoestrogen-Induced Transgenerational Inheritance**

Alterations in gene expression and subsequent phenotype can be induced by a variety of environmental exposures. It is thought that changes in the epigenome of oocytes or spermatocytes gives rise to these transgenerational effects (178, 179). A number of recent studies have demonstrated that many EDCs, including DES, methoxychlor, vinclozolin, and PCBs, can modify rodent phenotype, and that these modifications are heritable through many generations (180, 181). For example, Anway and colleagues (182) demonstrated that *in utero* exposure to methoxychlor or vinclozolin reduced fertility through altering DNA methylation patterns in rats. The authors found this phenotype to be inherited through F4 generation rodents. Similarly, vinclozolin appears to affect reproductive function and tumor incidence in female rats exposed to this EDC during gestation (183). While it appears that EDCs not only affects the generation of organisms immediately exposed to these chemicals, but subsequent generations of exposed organisms, this data remains very controversial (184).

## 1.4 XENOESTROGENS AND DEVELOPMENTAL ORIGINS OF HORMONE-DEPENDENT TUMORS

Endocrine tissues developing during the perinatal period are vulnerable to endogenous and exogenous stimuli due to the ongoing programming of the endocrine axis and development of the reproductive tract and accessory endocrine organs. However, the methods by which perturbation of hormone signaling during development increases hormone-dependent tumor risk is not well understood. A key to these early programming events is the endocrine signaling of hormones in the reproductive tract and feedback of hormones from the gonads or accessory glands to the hypothalamus and pituitary. Paracrine and autocrine signaling, such as discussed above, also play important roles in programming of endocrine tissue. During puberty in the mammary epithelium, for example, mammary differentiation is prevented via autocrine signaling from growth hormone (GH) (185). Importantly, tumors can supplant the requirement for exogenous mitogenic signals in order to transform normal paracrine-responsive prostate and breast tissue into neoplastic tissue (186, 187). During development, disruption of endocrine signaling by EDCs can have several effects: 1) they can generate premature, faulty or alternative endocrine signaling; 2) they can change the epigenetic landscape of the genome, which can increase the susceptibility of tissues to neoplastic transformation, especially after puberty when endogenous hormones stimulate proliferation and differentiation of reproductive tract tissues; and/or 3) they can change the normal hormonal milieu such that differentiation is disrupted or altered (169, 188).

Disruption of estrogen homeostasis or ER can negatively impact female reproductive tract development and result in development of adult reproductive tract neoplasias, as evidenced by results from *in utero* exposure to the xenoestrogen DES. E2 also plays important roles in metabolism and in coordinating the morphological alterations that occur during the menstrual cycle and pregnancy. Likewise, the exposure of an adult to abnormal levels of E2 or xenoestrogens can result in infertility and cancer. This problem, however, is amplified during development when the reproductive tract is still differentiating, because of the plasticity of the undifferentiated cells. It is known from the Barker studies of nutrient deprivation during development (discussed in section 1.1) that signals during development can alter susceptibility to

cardiovascular disease and diabetes. Similarly, signals of nutrient availability result in changes in glucocorticoid levels in the fetus, leaving an epigenetic hormonal “imprint” that determines the ability of the offspring to adapt to the future environment via DNA methylation of the glucocorticoid receptor (189). When there is a contrast or “mismatch” between the maternal environment and the environment of the offspring, it has been postulated that the plasticity of the epigenome may constrain the offspring to a phenotype that increases risk for adult disease (190, 191).

A key factor in controlling E2 exposure during development is the estrogen scavenger alpha-feto protein (AFP). AFP is produced by the liver until ~PND 16 in rodents and decreases precipitously after gestation week 14 in humans (192), which allows the reproductive tract to initially develop in an estrogen-independent manner. However, AFP does not usually recognize xenoestrogens, and so exposure to xenoestrogens during this critical time has been shown to developmentally reprogram the reproductive tract, or create a “mismatch” in future ER signaling of the offspring. This “mismatch” can result in permanent alteration of the adult response to endogenous estrogen (193), and subsequently increase the risk of reproductive tract neoplasias. Interestingly, in animal models where AFP has been deleted, the phenotypic result is similar to that of neonatal DES exposure, such that the mice are infertile and anovulatory (194). This evidence indicates that the reproductive tract environment during development is dependent upon estrogen concentrations.

*In utero* exposure to DES in humans is associated with an increased occurrence of multiple functional disorders, deformations and neoplasias of the female reproductive tract. These disorders include malformed uteri, infertility, vaginal clear cell carcinomas, endometriosis and potentially uterine fibroids. Exposure to DES, however, is just one of many EDCs that have been shown to be associated with disorders of the female reproductive tract. Epidemiological studies of adult exposure to organochlorines, such as TCDD, DDT, methoxychlor and PCBs, have shown a correlation between adult exposure and endometriosis in 4/10 studies (195). DES exposure *in utero* has also been correlated with an increased risk of endometriosis (80%; 1.8 relative risk) in “DES daughters” according to a report from the Nurses’ Health Study II (196). Additionally, animal studies of *in utero* or neonatal exposure to xenoestrogens have demonstrated a developmental reprogramming effect leading to multi-oocyte follicles and aneuploidy (upon exposure to DES, GEN or BPA). These reprogramming effects,

which negatively impact fertility and offspring, also result in endometrial neoplasia (from TCDD or DES) and uterine neoplasias (exposure to DES or GEN) such as uterine adenocarcinomas (35, 51) and leiomyomas (197). The evidence from all of these studies indicates that a “mismatch” can be created by inappropriate estrogen exposure that leads to an abnormal hormonal milieu and increased uterine neoplasias.

#### **1.4.1 Etiology of Uterine Leiomyoma**

Developmental reprogramming of uterine neoplasias has been demonstrated by exposure to xenoestrogens in humans and in animal studies. Specifically, DES exposure *in utero* in humans is correlated with an increased risk for uterine leiomyomas or fibroids (76% in exposed group vs. 52% in unexposed group with leiomyomas; odds ratio [OR] 2.4, CI 1.1-5.4) (29). Additionally, in women older than 35 years of age who reported “probable” or “definite” exposure to DES *in utero*, there was a significant correlation with increased incidence of uterine leiomyoma (47). However, this association remains controversial (198). Importantly, uterine leiomyomata are one of the most common gynecological tumors in women (77%) and the leading cause of hysterectomy among women of childbearing age (199). Uterine leiomyoma are monoclonal (200) hormone-dependent benign neoplasms that arise from the smooth muscle layer of the myometrium, and they are characterized by an excess secretion of extracellular matrix (ECM) proteins. Genetic abnormalities have been observed in about 40% of uterine leiomyoma, and they include translocations and deletions of chromosomes 6 and 7 that are associated with mutations in the genes high mobility group A2 (*HMGA2*), transforming growth factor beta (*TGFβ*), fumarate hydratase (*FH*) and high mobility group A1 (*HMGA1*) (201). However, no one common gene mutation has been linked in all uterine leiomyomas.

Hormone-dependency of uterine leiomyomas has been demonstrated by several studies, and have shown increased expression of both ER (202) and PR (203) as well as leiomyoma-specific increases in 17β-estradiol (204) and progesterone concentrations (205). Together with hormone dependency, several growth factors can mediate the mitogenic signals of estrogen and progesterone in uterine leiomyomas, including TGFβ, PDGF (206, 207), bFGF, EGF, IGF-1/IGF-1 receptor and IGF-II (208). Beyond the role TGFβ has in regulating differentiation and development, it also has

downstream effects. These effects include the modulation of expression of genes that result in ECM production and regulation, such as collagens, matrix metalloproteases (MMP) and tissue inhibitors of MMPs (TIMP) (209). Recently, it was found *in vitro* that TGF $\beta$  could activate Akt and repress PTEN through miRNA expression/activation, resulting in increased expression of the ECM protein Col1A2 (210). For fibrotic disorders like leiomyomas, tissue remodeling through ECM proteins is a key event in their progression. Since it has been shown that xenoestrogens modulate miRNA expression in breast progenitor cells (211), it is possible that they could also reprogram expression of miRNAs that control the expression of ECM proteins. This reprogramming of ECM proteins during development could, therefore, increase the risk of uterine leiomyoma.

Several factors that increase risk for uterine leiomyoma development have been identified, and they include nulliparity, early menarchy, age (women late in their reproductive years), ethnicity (e.g. African-American) and obesity (212, 213). Multiple epidemiological studies of risk factors associated with development of uterine leiomyomas clearly indicate a relationship between unopposed estrogen and tumor development. This relationship is supported by the increased risk with nulliparity and the decreased risk with multiple pregnancies, which has been demonstrated in both human and animal studies. Furthermore, the risk factor associated with early menarche, which increases time of exposure to unopposed estrogen, also supports this relationship. Another theory of uterine leiomyoma development proposes that progesterone may be more influential than estrogens (205), given that mitotic rates in leiomyomas are highest when progesterone peaks in the secretory phase of menses (214). In addition, the progesterone antagonist mifepristone (RU486) has also proven to be clinically effective at treating uterine leiomyomas.

The question of the initiating event for leiomyoma tumorigenesis, however, still remains outstanding. Given the widespread prevalence of this disease (i.e. the number of hysterectomies due to uterine leiomyoma) is 33% in the US (215), 68% in Nigeria (216), 47.6% in Malaysia (217), and 66.7% in France (218)), it seems the initiator event(s) would necessarily be shared by many women. As dictated by the principle of Occams razor, that is, the simplest answer is usually the correct explanation, the relationship between unopposed estrogen exposure and development of uterine

leiomyomas seems most likely to be the initiating circumstance. Compared to our evolutionary lifespan, in the last hundred years we live longer, have children later, have fewer children, have earlier menarche and have increased obesity incidence (associated with increased circulating estrogen), which makes the common denominator very likely to be unopposed exposure to estrogen (212). However, the predominance of progesterone in uterine leiomyoma development cannot be excluded. Extending from these theories, we may hypothesize that an early life exposure to xenoestrogens could create a hormonal milieu via developmental reprogramming of the reproductive tract that mimics the promotional effects of unopposed estrogen exposure.

#### **1.4.2 Rodent Uterus Development and the Eker Rat Model of Uterine Leiomyoma**

Given that estrogens drive the majority of female reproductive tract neoplasias, it logically follows that it would be important to determine if developmental exposure to estrogenic EDCs predispose the reproductive tract to neoplastic transformation. To understand the etiology of reproductive tract neoplasias, it is necessary to be familiar with the ontogeny of uterine development, which is species-specific and, as such, has different windows of susceptibility to EDC exposure. Unlike humans, who complete the majority of uterine development before birth, rodent uterine development and maturation is primarily completed after birth (219). Specifically, while Wolffian ducts regress, Müllerian ducts differentiate into the components of the female reproductive tract (vagina, oviduct, cervix and uterus) around gestational day 17. In the neonatal rat, the uterus differentiates from rudimentary mesenchyme and luminal epithelium into a more differentiated myometrium, with endometrial glands and circular myometrium present by PND 10. Between PND 10-15, both the longitudinal and circular layers of the myometrium, as well as the glandular endometrial stroma differentiation, are completed (220, 221). An important protein that regulates the amount of available estrogen in the fetus or neonate is alpha-feto protein (AFP), which, as discussed in section 1.5, binds endogenous estrogen and allows the uterus to develop in an estrogen-independent manner until ~PND 16 in rodents. This estrogen-independent uterine development correlates with hormone-independent development of the endometrial gland. This window when the fetus should be developing in a hormone-

free environment indicates that exposure to DES (which is not bound by AFP) before PND 16 may cause precocious and permanent gene expression alterations in the uterus, especially of estrogen-responsive genes. This response to xenoestrogens has been demonstrated in studies showing that neonatal exposure to DES in CD-1 mice during critical windows of uterine development (i.e. PND 1-5) permanently alters normal gene expression of *lactoferrin* and *c-fos*, and this persists in tumors (i.e. uterine adenocarcinomas) (170, 222, 223). Our lab has also shown the existence of a critical window of susceptibility, PND 3-12, for the development of uterine leiomyoma upon exposure to DES in the genetically susceptible Eker rat model.

While inheritance of gene defects is the strongest risk factor for cancer, environmental factors, including obesity, smoking, diet and exercise also contribute to cancer risk. Early development of malignant vaginal clear cell carcinomas only occurred in 1% of the DES exposed population, indicating the contributions of other risk factors, including the inheritance of gene defects. Differential penetrance or expressivity of disease phenotypes has been canonically attributed to factors such as modifier genes, environmental factors, allelic variation and gene-environment interactions (224). The contribution of both gene defects and environmental factors have been demonstrated to increase cancer risk in epidemiological studies. Inheritance of mutations in the tumor suppressor genes *BRCA1* or *BRCA2* are associated with development of breast and ovarian cancer. However, inheritance of these mutations demonstrates incomplete penetrance. King and colleagues (225) examined this issue using data from the New York Breast Cancer Study, which showed that there was a significant increase in risk for breast cancer in women born after 1940 as compared to those born before 1940. This study found a significant positive effect of exercise and body mass index (BMI) in adolescence with decreased breast cancer incidence in women carrying a *BRCA1/2* mutation. Interestingly, in a study comparing former non-athletes with former athletes, it was found that in former non-athletes, the relative risk (RR) of developing uterine leiomyoma was 2.53 (RR=1.86 for breast cancer) (226). This may be attributable to the protective benefits of college athletics, which includes lean body composition as well as dietary factors (227). A confounding factor in this study is the late age of onset of menarche as well as the prevalence of amenorrhea in female athletes, both of which would decrease the amount of exposure to unopposed estrogen. This information supports the importance

of the hormonal milieu in the risk of development of reproductive tract diseases, such as uterine leiomyomas, and its impact on penetrance of tumor suppressor gene defects.

One of the best-characterized models of uterine leiomyoma is the Long-Evans Eker rat model. The Eker rat, which carries a genetic defect, was the first animal model of renal cell carcinoma, and the gene defect was shown to be inherited in an autosomal-dominant fashion (228). The Eker rat model also was shown by our group to develop spontaneous uterine leiomyomas, in addition to the rare leiomyosarcomas and splenic hemangiosarcomas (229-231). The genetic alteration found to be associated with these tumors was a germline mutation identified as a retroviral insertion of DNA (5Kb) between exon 30 and 31 in the tumor suppressor gene *tuberous sclerosis 2* (*Tsc-2*). This mutation results in the expression of a 3' deletion of the GTPase-activating protein (GAP) domain of the TSC2 transcript (232, 233). The C-terminal GAP domain of TSC2 interacts with Rheb, a guanosine triphosphatase (GTPase), causing a conformational change to its GDP-bound "off" state. In this conformation, Rheb cannot mediate downstream activation of mammalian target of rapamycin (mTOR). In a recent study, examination of disease-associated TSC2 mutants revealed that nearly all had a deleterious effect on the GTP hydrolysis activity of Rheb (234). Furthermore, Eker rats that inherit two mutant copies of *Tsc-2* die between E11 and E13 (235), whereas inheritance of two wild type *Tsc-2* alleles produces no tumors in these animals (236, 237).

Using the Eker rat, we have been able to study the etiology of hormonally-driven uterine leiomyomas and the effects of neonatal xenoestrogen exposure. Eker rat uterine leiomyomas arise at a spontaneous rate of 65% by 16 months of age, although they are detectable as early as 10 months. This is similar to the incidence found in humans. In the Eker rat, as in humans, the majority of uterine leiomyomas arise from the smooth muscle of the myometrium, and are classified as either typical (fusiform), epithelioid or mixed. The leiomyomas are typically found at the uterocervical junction, although several have been found in the uterine horns, and rarely in the vagina. Additionally, the rare uterine leiomyosarcoma arises in the Eker rat (3/100), one of which was shown to metastasize to the lung, which is also the typical point of leiomyosarcoma metastasis in humans. Histologically, the Eker rat uterine leiomyomas are similar to human uterine leiomyomas. They are characterized by well-differentiated

smooth muscle cells that are fusiform and elongated in appearance (230). Like human uterine leiomyomas, Eker rat uterine leiomyomas also have a low mitotic index. Another characteristic of Eker rat uterine leiomyomas is the loss of tuberin expression in the majority of tumors, which is the protein expressed from the gene *Tsc-2*. Loss of tuberin expression has also been demonstrated in ~50% of human uterine leiomyomas (N=60 samples) (238). Additionally, in a microarray profiling analysis of human uterine leiomyomas, tuberin expression was decreased significantly (239). However, only ~50% of the uterine leiomyomas from Eker rats have shown loss of heterozygosity of the second wild-type allele of *Tsc-2*, indicating the wild-type allele may undergo mis-segregation, mitotic recombination, silencing or point mutagenesis (240-243). Additionally, it is possible that aberrant expression of miRNAs could lead to the loss of tuberin expression under altered hormonal milieu.

In addition to Eker rat uterine leiomyomas being histologically similar to human uterine leiomyomas, they are also similar in gene expression profiles. Using data from five studies (244-247) of gene expression profiles comparing human uterine leiomyoma to normal myometrium, we compared the gene expression profiles of genes differentially expressed in human and Eker rat uterine leiomyoma. Of these differentially expressed genes, 37/566 genes were found to be commonly altered in both species (**Table 1.3**) Similarly, Crabtree *et al.* (248), performed a microarray analysis using normal myometrium or uterine leiomyoma from human samples and from Eker rats. They also found that multiple genes (578/1543) were commonly differentially expressed in tumors from both human and rats, further supporting the importance of the Eker rat as a model of human uterine leiomyoma.

Human Gene Symbol	Eker N PE MvsPE T::fold change	Aha::fold change	Arslan::Average fold change	Hoffman::Fold Change	Tsibris::Mean	Wan
ALDH1A2	-2.49	-1.72				
ANPEP	4.89	-2.43				
C3	-32.61			-2.67		
CAPN6	3.32			3.42		
CCL11	-14.56	-5.61				
CCL21	-5.09	-3.94			-11.64	-8.5
CDKN1A	2.08	3.24			8.04	
CFH	-4.19				-5.34	
CKS2	2.77				3	
CLU	-4.51		-2.7	-1.99		
CRABP1	8.91				2.69	
CSPG2	4.28		3.4			
DIO2	6.87			4.68		
FOS	-2.97		-5.7	-9.52		
GATA6	-3.96					2
GPM6A	-2.25		-3.3			
GNPMB	3.68			-2.49		
GPR116	-3.86			-1.84		
GPX3	5.5			-2.4		
GRIA2	4.62			7.48	38.77	4.9
HOP	-2.86			3.03		
KCNF1	-8.54	-2.37				
KRT19	-11.75				8.09	
LEPR	-3.31			-2.22		
MAFB	2.86	2.26				
MGST1	3.28					-4.3
MMP11	2.87		5.8		5.07	
NCAM1	2.23			2.92		
NR2F2	-3.17			-2.36		
POPDC2	-3.58			3.11		
PTGER3	-6.4				-4.48	
S100A4	2.84		-3.2	-2.39		
SGK	3.65			-3.76		
SLC24A3	-2.62			2.27		
SMOC2	-2.19	-1.91				
SULF1	3.54			2.02		
TIMP3	-2.18		-2.9	-2.12		
Genes in common	37	8	7	19	9	4
# of DE genes	566	71	80	226	106	68

TABLE 1.3

While we know that inheritance of a tumor suppressor gene defect, such as BRCA1/2 or Tsc-2, increases the risk for cancer, less is known about the impact of environmental influences in individuals with genetic mutations on disease penetrance. Additionally, from multiple human and animal studies it has been shown that environmental estrogens can alter disease susceptibility in several different genetic backgrounds. We, as well as others, have demonstrated that one of the most susceptible windows for increased risk of reproductive tract disease from xenoestrogen exposure is during reproductive tract development. Given that DES can developmentally reprogram the reproductive tract in both humans and in genetically susceptible animal models of reproductive tract disease, we hypothesized that neonatal exposure to GEN and BPA developmentally reprograms the reproductive tract through changes in estrogen-responsive gene expression, which increases the incidence of uterine leiomyoma in Eker rats. The purpose of these studies herein were to determine: 1) which genes, specific to uterine leiomyoma, are candidates for developmental reprogramming by xenoestrogen exposure (**Specific Aim 1**); 2) can other environmentally relevant xenoestrogens, GEN and BPA, modulate tumorigenesis in the Eker rat model (**Specific Aim 2**); and 3) by what mechanism do xenoestrogens induce developmental reprogramming of the reproductive tract to confer an increased risk for uterine leiomyoma (**Specific Aim 3**).

**CHAPTER 2: IDENTIFICATION OF GENES ASSOCIATED WITH  
UTERINE LEIOMYOMA REPROGRAMMED BY NEONATAL  
EXPOSURE TO DIETHYLSTILBESTROL**

(Published Manuscript, *Reproductive Sciences*, 2008; Appendix A)

## **2.1 PURPOSE OF STUDY**

The ability of developing tissues to take cues from the environment, while important for normal patterning, leave it susceptible to aberrant programming leading to abnormal physiological responses in adulthood. Developmental reprogramming of disease, or the fetal origins of adult disease, as coined originally by Barker *et al.*, has been a demonstrated phenomenon for multiple diseases that have origins *in utero*, including diabetes and cardiovascular disease (1, 249). Several environmental factors, such as maternal diet, hypoxia and anthropogenic chemicals, can lead to developmental reprogramming of the reproductive tract (250, 251). In addition, the effect of aberrant environmental stimuli can alter the normal morphological program and hormonal milieu, leading to neoplastic transformation and reduced fertility (5, 252), which is best exemplified by development of vaginal clear cell carcinoma after *in utero* exposure to DES (27).

The etiology of uterine leiomyoma is not well understood. However, the increased risk of uterine leiomyoma in some cohorts of women exposed to DES supports the theory that xenoestrogens affect developmental reprogramming, which leads to diseases of the reproductive tract. Using the genetically susceptible Eker rat, our previous studies also support the relationship between early life exposure to DES and increased incidence of uterine leiomyoma (197). In order to elucidate the mechanism by which DES elicits its developmental reprogramming effects that results in increased tumor incidence, we conducted gene expression profiling. The purpose of this study was to identify estrogen-responsive gene targets of developmental reprogramming by xenoestrogens, which are associated with uterine leiomyoma development. Identification of genes that are susceptible to aberrant estrogen exposure is key to understanding the mechanism of developmental reprogramming and the etiology of uterine leiomyoma.

## **2.2 METHODOLOGY**

### **2.2.1 Animals and Treatments**

Eker rats from a closed colony at MD Anderson Cancer Center, Science Park-Research Division were cared for in accordance with the study protocol approved by MD Anderson Animal Care and Use Committee. Eker rats were given water and standard rat chow (Harlan Teklad 22/5 (7022) Rodent Diet, Houston, TX) *ad libitum* and were maintained on a 14:10 light-dark cycle, which is historically associated with a 65% tumor incidence in this animal model. The reprogramming effects of xenoestrogen exposure was examined after treating neonatal Eker rats on three consecutive days, PND 10-12, with 1µg/g body weight of DES [(Sigma Chemical Co., St. Louis, MO) in sesame oil] using a total of 50 µl of sesame oil vehicle per s.c. injection. A separate group of animals were given 50 µl of sesame oil injections as vehicle controls (VEH). For microarray analysis, 16-month-old Eker rats were sacrificed via CO<sub>2</sub> asphyxiation for tumor and myometrium collection. For developmental reprogramming analysis, 5-month-old animals were sacrificed for myometrium collection to determine reproductive tract morphology and isolate RNA for gene expression analysis. Analysis of estrogen response in neonatal uteri was evaluated by sacrificing (cervical dislocation) PND 12 animals 6 hours after the last of 3 DES or VEH injections given consecutively on PND 10-12.

### **2.2.2 Tissue Collection and Histology**

From rats sacrificed at 5 and 16 months, vagina, ovaries and uterus were removed and a section of the uterus (upper right horn) was taken for histology. The remaining tissue (tumors and whole neonatal uteri or adult myometrium) were snap frozen in liquid N<sub>2</sub> or fixed in 10% neutral buffered formalin (NBF) for 24-48 hours and then transferred into 70% ethanol before paraffin embedding, sectioning (6 µm) and staining. Sectioned ovaries, uteri and vagina were stained with hematoxylin and eosin for analysis of estrus or stage of reproductive senescence in 5 month old and 16 month old animals, respectively, prior to gene expression analysis. For gene expression analysis, uteri from adult animals were scraped with a sterile scalpel in cold PBS to obtain myometrium free of endometrium, and along with tumor tissue, snap frozen in liquid N<sub>2</sub> and stored at -80°. In order to obtain adequate amounts of tissue for RNA extraction from neonatal animals, 3 uteri were pooled together per sample. Also, cardiac blood was taken from 5 and 16 month old animals and allowed to clot at 4°C

overnight, and then the serum was separated by centrifugation at 4°C (1500 X g) for 10 minutes and stored at -20°C until analyzed for hormone (E2 and P4) levels.

### **2.2.3 Histological Staging of Estrous**

Staging was performed in accordance with the procedure described by Cook *et al.* (197). Briefly, the stage of estrus or reproductive senescence was determined by histological examination of ovary, vagina and uterine tissues of 16 and 5-month-old rats. For 16-month-old rats, they were classified by stage of reproductive senescence [Pseudo Pregnant (PP), Persistent Estrus (PE) or Anestrus (AN)], as determined by histological examination of ovaries, vagina and uteri. The stage of reproductive senescence classified as PP is defined as have a mucified vaginal epithelium and enlarged ovarian corpora lutea. The PE stage is defined as having a cornified vaginal epithelium and multiple ovarian follicles; whereas animals in the AN stage have atrophic ovaries and senescent vaginal epithelium. For 5-month-old VEH rats, stage of estrus was categorized into proliferative [proestrus (Pro) and estrus (E)] or secretory [metestrus (Met) and diestrus (Di)] phases as determined by histological examination of ovaries, vagina and uteri. Animals in proestrus have a keratinized vaginal epithelium, which begins to slough off during estrus. During metestrus the vagina becomes muscified and inflammatory cells are present in the uterus; whereas in diestrus the lumen of the uterus (sagittal section) appears collapsed. For DES-treated rats that do not cycle normally due to disrupted ovarian function (197), estrogen and progesterone levels were determined via radioimmunoassay (RIA), obtained using the Ultra-sensitive Estradiol and Active Progesterone kits from Diagnostic Systems Laboratories (Webster, TX) according to the manufacturer's protocol, and as described by Cook *et al.* (197). Following the RIA assay all tubes were inverted and allowed to dry for 2 hours. All tubes were then counted on the gamma radiation counter for 1 minute. After RIA analysis, DES-treated rats were grouped into either high estrogen and progesterone levels (High E/P) and compared to proliferative phase animals, or low estrogen and progesterone (Low E/P) for comparison to secretory phase animals.

### **2.2.4 Microarray Analysis**

RNA from tumors and normal myometrium of 16-month-old Eker rats was isolated as described previously (197) and analyzed using an Affymetrix Chip Rat 230 2.0 array. Analysis of the array was conducted using the Gene dChip program with confidence interval set at 95% and differences in fold change in gene expression were significant at  $p < 0.05$ .

### **2.2.5 Microarray and Statistical Analysis**

For analysis of the gene expression changes from the Gene Chip Rat Genome 230 2.0 array, raw signal intensities for each probe set as they are contained in the CEL files were analyzed using Chen's dChip (<http://biosun1.harvard.edu/complab/dchip/>) model-based expression analysis to obtain significant genes with fold changes of  $>1.5$  or  $<-1.5$ , and a false discovery rate  $<0.4\%$  of 50 permutations. After obtaining model-based expression values, both biased and unbiased hierarchical clustering high-level analysis was performed using dChip. For statistical analysis of real-time PCR data, a linear model analysis was applied to estimate the interaction effects of gene and treatment.

### **2.2.6 Microarray Validation and Real-Time PCR (qPCR)**

Validation of microarray data was conducted by real-time PCR of cDNA from identical samples (if available) or comparable age and stage matched myometrium and tumors. Frozen tissue (tumors or myometrium) from 16 month old animals were crushed under liquid nitrogen with mortar and pestle and RNA was isolated after DNA removal, according to protocol for Ambion's RiboPure™ Kit (Austin, TX). Following RNA extraction, cDNA was made by reverse-transcribing 1  $\mu$ g of RNA using the Invitrogen Superscript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). Aliquots of cDNA were made for each sample and stored in a 1:10 dilution of tRNA water at  $-20^{\circ}\text{C}$  until analyzed. Real-time PCR was performed using the 7900T Fast Real-Time detection system from Applied Biosystems (ABI, Foster City, CA). Fast Real-Time Taq-Man assays from ABI were used to analyze gene expression of *Gdf10*, *Cspg2*, *Car8*, *Calbindin D9k*, *Vcam1*, *Kcnf1*, *Rasd2*, *Sfrp2*, *Tacstd1*, *Krt19*, *Rps6kb1*, *Nr2f2*, *Gria2*, *Igfbp5*, *Spp1*, *Dio2*, *Aqp3*, *Ramp3* and *Mmp3*.

All real-time PCR reactions were performed by first mixing Universal Fast Real-Time Master Mix from ABI with the gene assay mix, and then adding 2  $\mu$ l of cDNA from each sample to make up a 25  $\mu$ l volume. For an endogenous control, glyceraldehyde-3-phosphate (GAPDH) was used, which included probe and forward and reverse primer in a 25  $\mu$ l reaction volume. The following set of conditions were used for each real-time reaction: 95°C for 10 minutes followed by 40 cycles at 95°C for 1 second and 60°C for 20 seconds. The real-time PCR reactions were all performed in triplicate and were quantified using the  $-\Delta\Delta C_T$  method, which uses the average  $C_T$  of the GAPDH subtracted from the target gene  $C_T$  to obtain the average  $\Delta C_T$ . A calibrator from each set of samples was chosen from which to subtract individual VEH and DES sample  $\Delta C_T$  values to obtain the  $-\Delta\Delta C_T$ . The fold change for each sample was calculated in comparison to the calibrator by taking  $2^{-\Delta\Delta C_T}$ . The calibrator for both 16 month and PND 12 rats was VEH treated myometrium or uteri. For samples from 5-month-old rats, estrous VEH treated myometrium was used as the calibrator.

## **2.3 RESULTS**

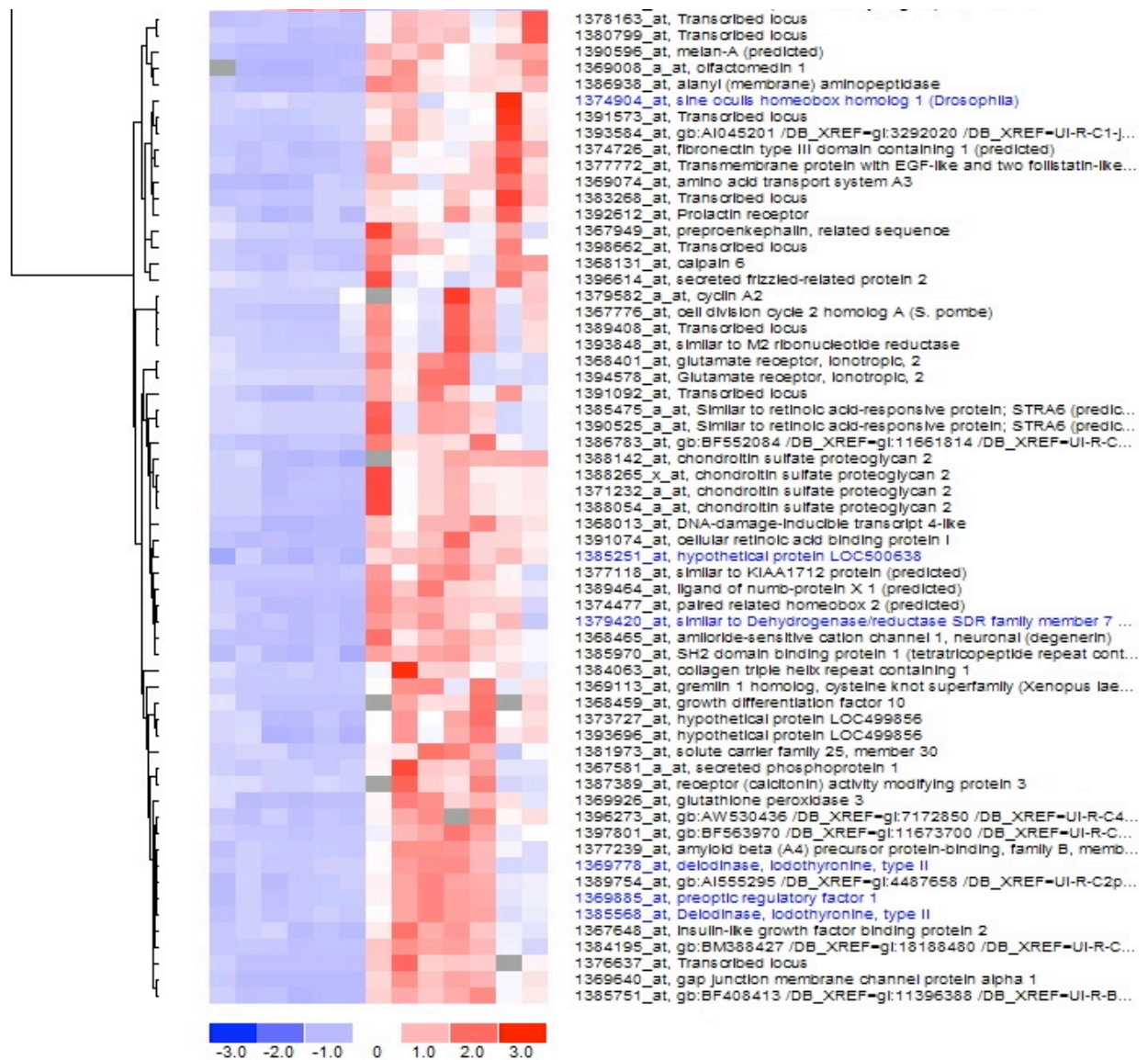
### **2.3.1 Discovery of Candidate Genes Associated With Uterine Leiomyoma and Developmental Reprogramming in Eker Rats**

In order to obtain candidate genes for DES-induced developmental reprogramming in the uterine myometrium, a cDNA microarray analysis was performed using the Affymetrix Rat 230 2.0 array. To control for hormonal influence and age, all tissues used for analysis were matched according to stage of reproductive senescence and age. Stage of reproductive senescence was classified by histological examination into AN, PP or PE (as described in the Methodology section). Eker rats historically demonstrate a 65% incidence of uterine leiomyoma by 16 months, so myometrium or tumors were obtained from animals at this age and RNA was isolated from each tissue sample for microarray analysis.

After generation of raw gene expression data, dChip was used to determine clustering hierarchy in both a supervised and unsupervised method. Unsupervised clustering revealed a distinct differential expression profile between normal myometrium and tumors from Eker rats. Furthermore, within the tumor samples, which represented both tumors from unexposed or DES-exposed Eker rats, hierarchical

clustering demonstrated a distinction between tumors from DES-exposed and unexposed animals (**Figure 2.1**). These data indicate that neonatal DES exposure changes the expression profile of uterine leiomyomas from that of spontaneous tumors that develop in Eker rats.





**Figure 2.1 Representative heat map of microarray analysis.** Heat map represents unsupervised comparison of VEH PE myometrium and DES PE tumors from 16mo. old animals.

Given that uterine leiomyomas are hormonally-driven tumors that demonstrate dependence on estrogen and progesterone for sustained growth, we chose to focus on those differentially expressed genes that contained estrogen responsive elements (as determined by *in silico analysis*). These were the primary candidate genes for developmental reprogramming by DES. Using the algorithm in the Dragon Estrogen Responsive Element v 2.0 program (<http://sdmc.lit.org.sg/ERE-V2/index> (no longer available)) (253), which utilizes canonical and non-canonical ERE patterns to identify known or putative EREs in genes, we identified 112/171 (65%) of the differentially expressed genes as estrogen-responsive. Specifically, the analysis revealed 86 genes with a putative ERE, and 26 genes with a known functional ERE. The large number of differentially expressed genes that were identified as estrogen-responsive in tumors as compared to normal myometrium demonstrates the hormone-dependence of uterine leiomyoma development. Examples of the genes identified *in silico* as containing EREs are listed in **Table 2.1**.

TABLE 2.1

<b>Table 1</b>	<b>Selected Representative ERE Containing Genes Identified by Dragon 2.0</b>		
<b>Gene</b>	<b>Predicted ERE (Forward)</b>	<b>Predicted ERE (Reverse)</b>	<b>ERE location relative to the 5' end of the gene</b>
Dio2		GT-GGTCA-TCT-CAACC-CT	R=-9376
CalbindinD9k	CA-GGTCA-GGG-TGATC-TT	AA-GATCA-CCC-TGACC-TG	F=50; R=-66
	AG-GGTCT-ATG-TAGCC-CA		F=1507
GRIA2	GA-GGTCA-GTC-TGATC-AA	GG-GGGCA-TTG-TGACC-CT	F=19005; R=-51017
	AA-AATCA-AAT-TGACC-CC	CC-AGTCA-TTC-TGCCC-AA	F=19719; R=-2244
CAR8	GT-AGTCA-CAC-CAACC-CT	TG-GGTCC-ACT-TGCCC-TC	F=33744; R=-38190
	CA-AGTCA-AAA-CAACC-CT	AA-GGTGG-CTT-TGTCC-AG	F=45678; R=-9263
GDF10		AT-GGACA-ATA-TGACC-TT	R=-10503
		AT-GGTCA-AGA-TGCCT-GA	R=-10442
		AG-GCTCA-CAC-TGCCC-TC	R=-7249
MMP3		GT-AGTCA-CCA-CACCC-TT	R=-12519

After identification of estrogen-responsive genes, we chose 19 of these genes that seemed to have the most biological relevance to uterine leiomyoma for validation against the results from the microarray analysis. qPCR analysis of these 19 genes showed 18/19 to be valid in differential expression using identical samples (when available) from the cDNA microarray (**Table 2.2**). The 18 validated estrogen-responsive genes were used for the remainder of the study as a training set of candidate genes for further analysis of developmental reprogramming by DES in neonatal and adult myometrium.

TABLE 2.2

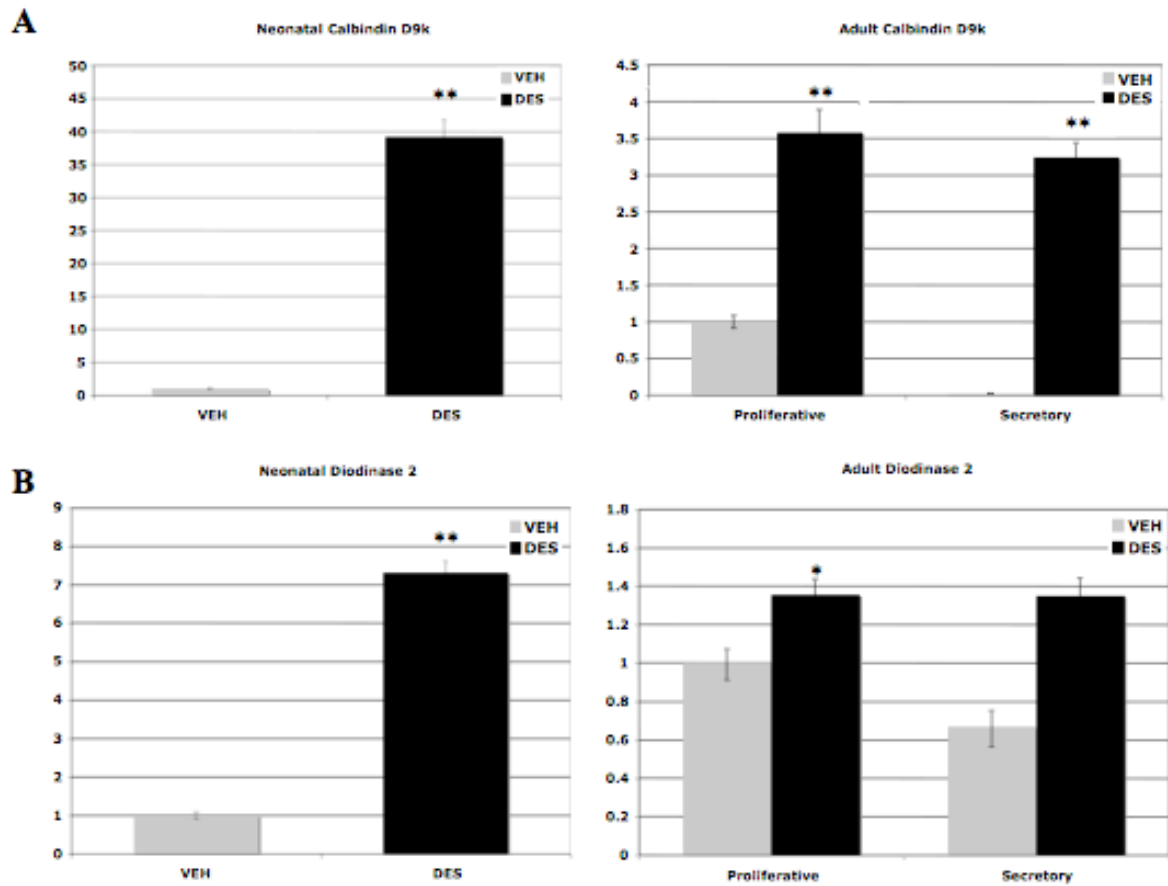
Gene	FC-Array	FC-Real-Time PCR
Gdf10	6.73	19.97
Cspg2	3.33	1.99
Car8	4.34	2.04
Calbindin D9k	9.63	28.05
Vcam1	2.31	-1.25
Kcnf1	2.51	1.74
Rasd2	4.77	11.47
Gria2	37.02	17.53
Dio2	5.23	3.43
Mmp3	5.32	38.20
Sfrp2	6.57	7.06
Tacstd1	-12.87	-177.30
Krt19	-12.63	-440.20
Rps6k	-6.36	-2.07
Nr2f2	-3.96	-2.11
Igfbp5	-3.32	-8.65
Spp1	-3.24	-1.46
Aqua3	-4.08	-16.39
Ramp3	-5.53	-11.31

(note: all samples run in triplicate), FC = fold change

### 2.3.2 Developmental Reprogramming of Estrogen-Responsive Genes by Neonatal Exposure to DES

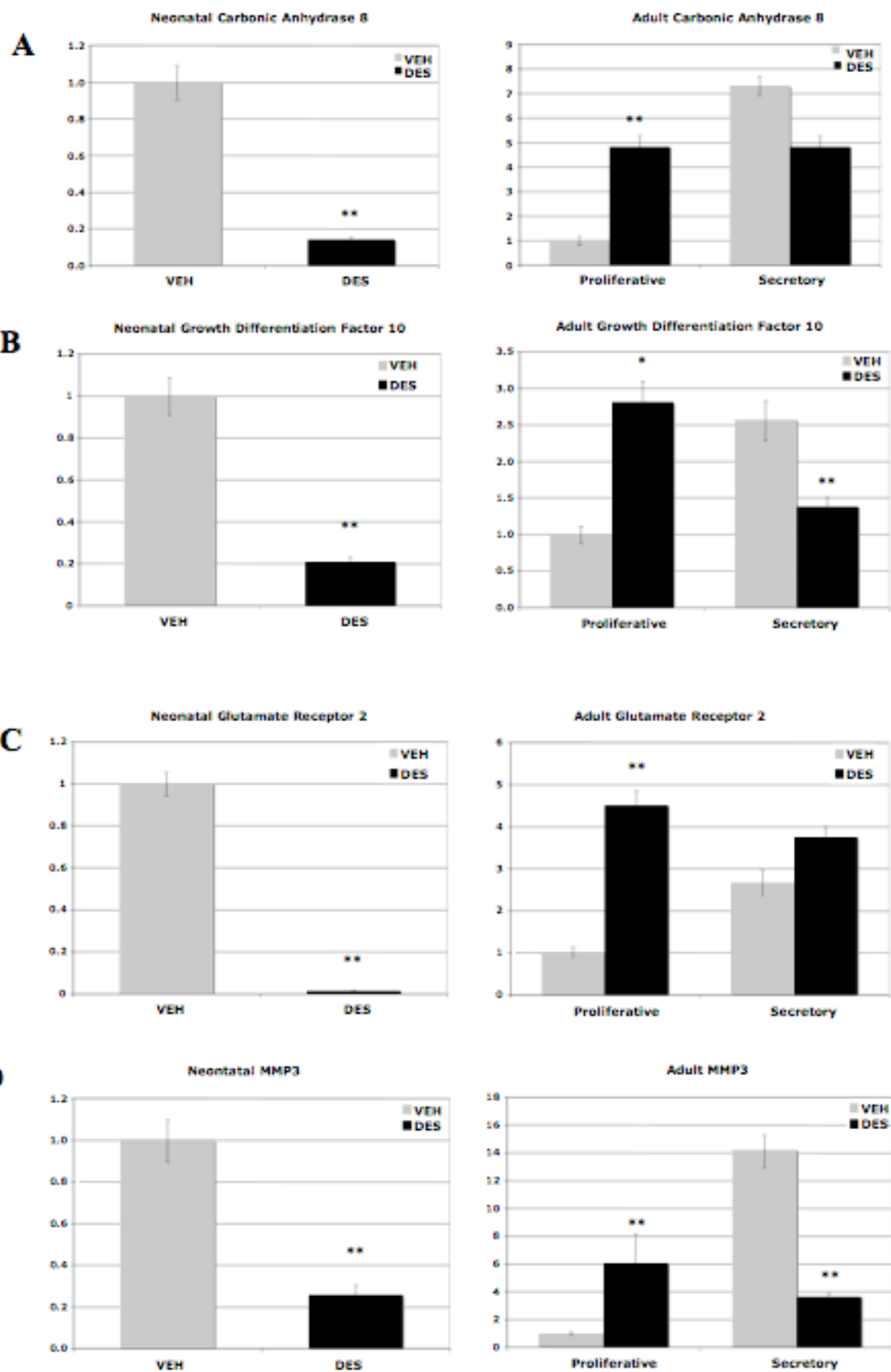
Developmental reprogramming of candidate genes were analyzed for 1) their response to DES exposure immediately after treatment (e.g. 6 hours after the last injection on PND 12); and 2) the persistence of gene expression changes in the adult myometrium from DES-exposed or control rats. We characterized genes as developmentally reprogrammed if gene expression was responsive to estrogen neonatally, and subsequently if expression was significantly different in DES exposed myometrium in comparison to VEH (control) animals in stage and age-matched adult myometrium. As demonstrated previously by our laboratory, neonatal exposure to DES induces a robust reprogramming effect, which results in lack of normal estrus in these animals (197). Hence, in order to compare gene expression accurately in VEH and DES animals, estrogen and progesterone levels were measured and grouped into either high estrogen/progesterone (High E/P) or low estrogen/progesterone (Low E/P), which would be concordant with the proliferative (proestrus/estrus) phase and the secretory (metestrus/diestrus) phase, respectively. These groups were then stage and age-matched (5 months of age) to VEH animals for analysis of developmental reprogramming of gene expression.

Upon analysis of candidate genes for developmental reprogramming we found that DES initially induced precocious expression of genes that are normally either induced or repressed after puberty by endogenous estrogen. These genes fell into two categories: a) genes that were induced in their expression immediately after neonatal DES exposure and whose adult expression became hyper-responsive to estrogen; and b) genes that were repressed immediately after neonatal DES exposure and whose expression was significantly induced in the proliferative phase as compared to VEH. Those genes that fell into first category included one of two genes that we had previously identified as developmentally reprogrammed by neonatal DES exposure, *Calbindin D<sub>9k</sub>* (254), which served as an internal control for this study. The other gene with a similar reprogramming profile was *Dio2*, which was significantly induced in expression by DES exposure neonatally and remained induced by endogenous estrogen in the proliferative phase (**Figure 2.2**).



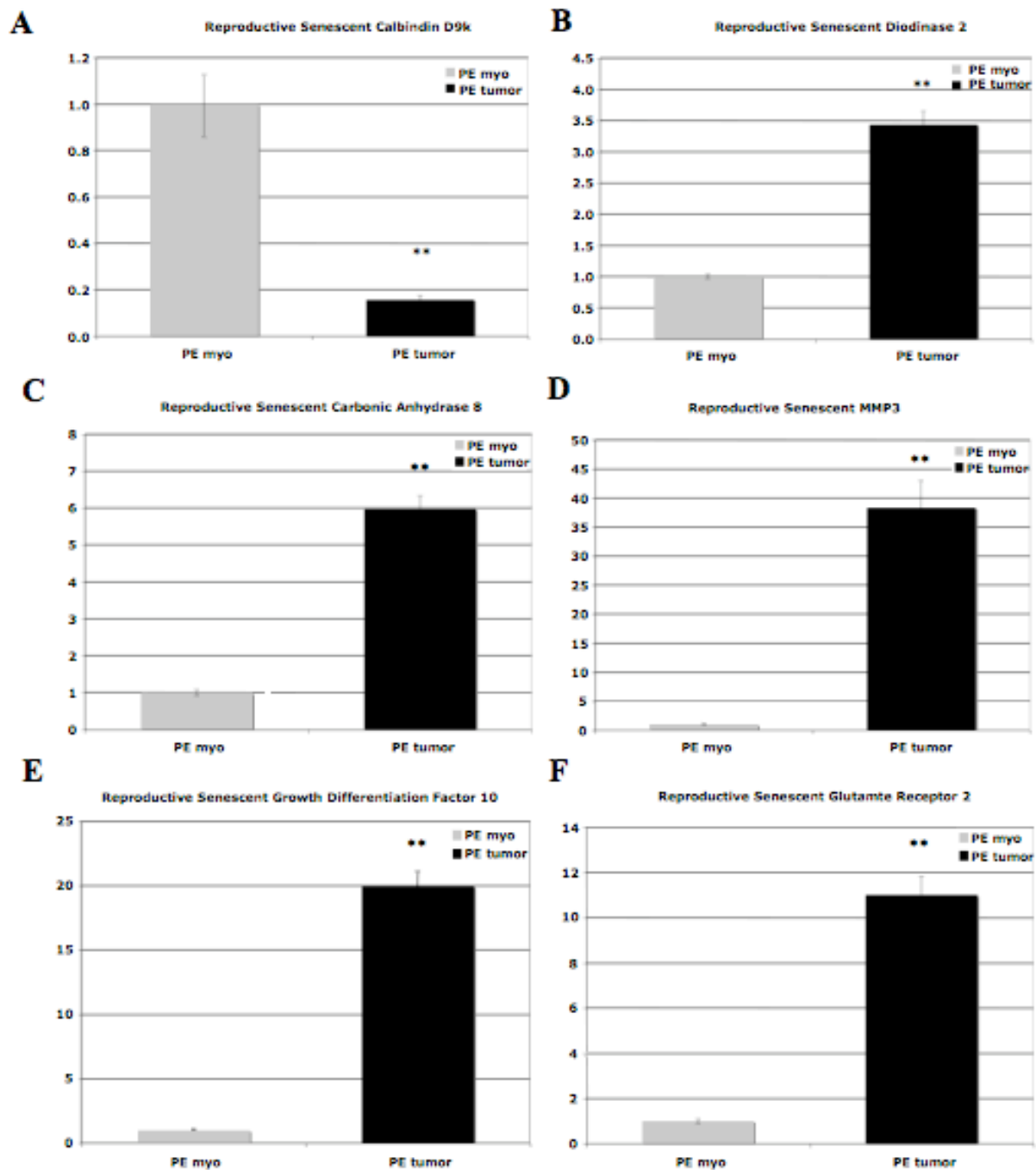
**Figure 2.2 Real-time RT-PCR for DES-reprogrammed genes induced by hormone in uteri (PND 12) (left panel), and myometrium (5 months) (right panel). A-B) Gene expression for *Calbindin D9k* and *Diodinase 2 (Dio2)* at PND 12 (VEH n = 9, DES n = 9), 5 months (VEH = 5, DES = 5). \*\* =  $p \leq 0.001$ , \* =  $p \leq 0.05$ .**

Genes whose developmental reprogramming profile fell into the second category of initial repression followed by hyper-responsiveness in the adult myometrium, included *Car8*, *Gdf10*, *Gria2* and *Mmp3* (**Figure 2.3**). This striking difference between the initial repression of gene expression, which mimicked the adult response, was reversed by DES exposure. Specifically, in the adult exposed to DES neonatally the response to endogenous hormones was opposite of those not exposed to DES, which caused an increase in expression in the proliferative phase rather than repression. These data reveal an important mechanism of DES reprogramming. Regardless of whether the estrogen-responsive gene was normally repressed or induced by endogenous estrogen, the effects of neonatal DES exposure was to induce a hyper-responsiveness in the adult myometrium as compared to VEH animals.



**Figure 2.3 Real-time RT-PCR for DES-reprogrammed genes repressed by hormone in uteri (PND 12) (left panel), and myometrium (5 months) (right panel).**  
A-D) Gene expression for carbonic anhydrase 8 (*Car 8*), growth differentiation factor (*Gdf10*), glutamate receptor 2 (*Gria2*) and matrix metalloprotease 3 (*Mmp3*) at PND 12 (VEH n = 9, DES n = 9), and 5 months (VEH = 5, DES = 5). \*\* =  $p \leq 0.001$ , \* =  $p \leq 0.05$ .

In order to determine if these developmentally reprogrammed genes were persistent in their overexpression in uterine leiomyoma from Eker rats, we compared the expression of reproductively senescent myometrium to tumors in age (16 months) and stage-matched (PE) animals (n=8) via qPCR. Importantly, 5/6 of the genes (*Gdf10*, *Car8*, *Gria2*, *Dio2*, and *Mmp3*) identified as developmentally reprogrammed by neonatal DES exposure in the adult myometrium were found to be overexpressed in tumors (n=4) as compared to senescent myometrium (n=4) (**Figure 2.4**). These data reveal a potential mechanism by which DES induces an increased susceptibility to and an increased incidence of uterine leiomyoma. DES induces a hyper-responsive state in estrogen-responsive genes in the adult myometrium that are commonly upregulated in uterine leiomyoma. Unlike VEH-treated animals, DES developmentally reprogrammed gene expression in the myometrium that correlated with uterine leiomyoma formation, thereby altering the normal hormonal milieu in such a way as to create an enhanced environment for uterine leiomyoma development.



**Figure 4. Real-time RT-PCR for reprogrammed genes differentially expressed in PE tumors vs PE myometrium from reproductively senescent Eker rats. A-F)** Gene expression for *Calbindin D9k*, deodinase 2 (*Dio2*), carbonic anhydrase 8 (*Car 8*), matrix metalloprotease 3 (*Mmp3*), growth differentiation factor (*Gdf10*), and glutamate receptor 2 (*Gria2*) (PE myometrium n=2, PE tumors n=8) \*\* = p≤0.001, \* = p≤0.05.

## 2.4 DISCUSSION

Xenoestrogens, including DES, have been shown to induce reproductive tract malformations via developmental reprogramming, leading to neoplastic transformation. *In utero* DES exposure, as demonstrated in epidemiological studies (29, 47), is associated with the highly prevalent reproductive tract disease uterine leiomyoma, indicating that early life exposure to xenoestrogens is a risk factor for development of this disease. In our previous studies we have also shown that early life DES exposure, in the presence of a tumor suppressor gene defect, increases uterine leiomyoma incidence to 100% (197) when exposure occurs during the critical development period for rodent uterus formation (PND 3-12) (254). While multiple studies have identified gene defects in uterine leiomyoma, little is known about the mechanisms of developmental reprogramming in the reproductive tract that gives rise to increased incidence of uterine leiomyoma. To address this gap in knowledge, we undertook a gene expression profiling study to identify genes in the myometrium that are targets of reprogramming by DES. Comparing uterine leiomyomas and myometrium, we selected 18/171 estrogen-responsive genes as candidates for developmental reprogramming by DES. Expression of 6 of these genes (*Gdf10*, *Car8*, *Calbindin D9k*, *Gria2*, *Dio2* and *Mmp3*) (255) were found to be reprogrammed by neonatal exposure to DES in the myometrium, 5 of which, *Gdf10*, *Car8*, *Gria2*, *Dio2* and *Mmp3*, retained an elevated level of expression in tumors. This increased expression was identified in tumors from both DES-exposed as well as unexposed animals, indicating that DES induces developmental reprogramming of estrogen-responsive genes that associated with genes that are normally differentially expressed in uterine leiomyoma.

In concordance with studies that show the dependence of uterine leiomyoma growth on estrogen, our gene expression analysis also demonstrated that the majority (65%) of genes found to be differentially expressed in tumors were either known or predicted estrogen-responsive genes. In several transcriptional profiling studies (222, 256, 257), DES has demonstrated the ability to induce or repress the expression of estrogen-responsive genes *in vitro* (MCF7 cells) and *in vivo* (neonatal rat uteri). In addition, Hong *et al.* also identified *Calbindin D9k* to be induced by DES exposure in immature rat uteri, which supports our findings (258). A hallmark of DES exposure that we found in our study was its ability to induce the persistent expression of genes both

increased and repressed normally by endogenous estrogens. These findings indicate that DES induces a hyper-responsiveness, given that developmentally reprogrammed genes were expressed even during phases of estrous characterized by low estrogen levels (i.e. secretory phases; metestrus/diestrus). Importantly, in CD-1 mice susceptible to uterine adenocarcinoma, Newbold *et al.* (169) found that neonatal exposure to DES induced the persistent elevation of estrogen-responsive genes associated with tumor development. This study together with ours indicates that in estrogen-driven tumors, the effect of neonatal DES exposure is to reprogram gene expression in the uterus/myometrium of genes that are important in tumor development. These data suggest that DES leaves a “hormonal fingerprint” that alters the normal hormonal milieu via differential gene expression, leading to increased uterine tumor development.

The mechanisms by which developmental reprogramming of estrogen-responsive genes occurs has yet to be fully elucidated. Several studies, however, have provided evidence to suggest that epigenetic modification may be playing a role in gene expression alterations leading to physiological changes in estrogen response. In particular, the perturbation of DNA methylation in estrogen-responsive genes has been demonstrated as a mechanism of neonatal DES exposure. Li and colleagues (171), demonstrated that the estrogen-responsive gene *c-fos* becomes hypomethylated at Exon 4 and subsequently remains overexpressed in the adult uterus after neonatal DES exposure. Interestingly, the hypomethylation was most apparent by PND 17, which corresponds with the decrease in the estrogen-binding protein AFP and the increase in estrogen in the uterus. This indicates that the epigenetic programming response to estrogen changes if DES exposure occurs during development. Similarly, in a previous study by the same group, they showed that the estrogen-responsive gene *lactoferrin* was also persistently up-regulated, which corresponded to site-specific DNA hypomethylation after neonatal DES exposure in the uterus (170). Furthermore, they illustrated the critical importance of adult endogenous estrogen production on establishing DES-induced changes in DNA methylation, given that only in the mature uteri was *lactoferrin* shown to be aberrantly demethylated. Importantly, this aberrant methylation was also sustained in uterine tumors from mice exposed neonatally to DES. Recently, Tang *et al.* (174) provided evidence that the estrogen-responsive chromatin remodeling gene *Nsbp1* undergoes

promoter hypermethylation immediately after DES exposure. However, upon exposure to adult hormones this pattern reversed, leading to hypomethylation of *Nsdp1* and over-expression that persisted in adulthood and corresponded to the formation of uterine tumors. These data indicate that DES triggers an epigenetic event in estrogen-responsive genes that becomes unmasked in certain genes by adult hormone exposure, which permanently changes the expression of the gene and potentially the susceptibility to uterine tumor formation.

In conclusion, the results of this study show that DES exposure during a critical period of formation for the uterus in rodents developmentally reprograms expression of estrogen-responsive genes associated with uterine leiomyoma formation. As will be demonstrated in Chapter III, the xenoestrogen genistein also modulates expression of estrogen-responsive genes and correlates with tumorigenesis, which supports the results of this study (Table II.3). The importance of these findings are relevant to humans because multiple genes identified in this study are also increased in expression in human uterine leiomyomas. The identification of genes reprogrammed by DES prior to tumorigenesis that are also up-regulated in human leiomyoma indicate that these genes may harbor features that make them susceptible to alteration during development by premature estrogen exposure. Additionally, elucidating the mechanism of DES-induced developmental reprogramming of gene expression in the reproductive tract will facilitate the process of defining the relationship between environmental endocrine disruptors, such as xenoestrogens, and increased risk of uterine leiomyoma.

**CHAPTER 3: XENOESTROGEN-SPECIFIC MODULATION OF NON-  
GENOMIC SIGNALING THROUGH HISTONE METHYLTRANSFERASE,  
EZH2, CORRELATES WITH DEVELOPMENTAL REPROGRAMMING  
OF GENE EXPRESSION AND TUMORIGENESIS**

### 3.1 STUDY PURPOSE

The term “developmental reprogramming”, as described in chapter 1, is used to describe the effects of early life exposures to adverse stimuli that can alter the response to normal physiological signals and give rise to disease in adulthood. Numerous studies, including those from our laboratory, demonstrate that perinatal exposure to xenoestrogens, many found ubiquitously in the environment, can developmentally reprogram the female reproductive tract, causing alterations in morphology, hormonal milieu, and gene expression, which can give rise to diseases such as infertility and cancer, as described in chapter 1 and illustrated in chapter 2 (41, 51, 252, 259-261). Developmental reprogramming of the reproductive tract by exposure *in utero* to the xenoestrogen diethylstilbestrol (DES) was an early example of this phenomena.

Although DES is no longer in clinical use, other environmental xenoestrogens have the potential to affect the developing reproductive tract and induce developmental reprogramming. For example, bisphenol A (BPA) can induce morphological abnormalities of the reproductive tract in rodents exposed neonatally to this xenoestrogen (69, 71, 72, 74, 77, 262, 263). BPA can cause precocious puberty, persistent vaginal cornification, lack of corpora lutea, cystic ovaries, cystic endometrial hyperplasia, and polyovular follicles in adult animals exposed perinatally to this compound (76, 77, 264, 265). Importantly, neonatal exposure at environmentally relevant micromolar doses has been shown to promote neoplastic transformation of the male reproductive tract, including formation of prostatic intraepithelial neoplasia (266). Genistein (GEN) can also induce developmental reprogramming of the female reproductive tract in animals. As described in chapter 1, multiple studies report that neonatal exposure to environmentally relevant doses of GEN (e.g., 2.4-6.6  $\mu$ M in plasma) (48) aberrantly affects reproductive function and morphology as evidenced by induction of ovarian and uterine morphological abnormalities, persistent estrus, accelerated vaginal opening, infertility, early reproductive senescence, multiocyte follicles, and uterine adenocarcinomas (51, 54, 56). Importantly, in a recent epidemiological study of over 19,000 women, a correlation was found between early life soy formula consumption and increased risk of uterine leiomyomas (267), further supporting the relevance of this study to the human disease.

While uterine leiomyoma are the most frequent gynecologic tumor of women, little is known about how environmental exposures may contribute to the high incidence of this disease. Previous studies from our group have demonstrated that, in genetically predisposed Eker rats, susceptibility to the development of uterine leiomyoma is modulated by developmental exposure to DES via developmental reprogramming (197, 268). In these animals, DES also reprogrammed the morphology of the reproductive tract, giving rise to persistent vaginal cornification and ovaries that lacked corpora lutea. And as demonstrated in chapter 2, microarray analysis identified several estrogen-responsive genes developmentally reprogrammed by neonatal DES exposure, which became hyper-responsive to hormone in the uteri of adult animals prior to the onset of tumors (269).

The mechanism(s) by which xenoestrogens, such as DES, induce developmental reprogramming of gene expression, however, is not well understood. The canonical pathway of genomic activation by estrogen receptor (ER) is well defined and can be induced by both steroidal ( $17\beta$ -estradiol) and non-steroidal chemicals (xenoestrogens). In contrast, the non-canonical pathway of rapid ER signaling is less well understood, though several studies demonstrate that rapid, non-genomic ER signaling via mitogenic pathways, including Akt and MAPK, is important for blood vessels vasodilatation, neuron survival, bone turnover and reproductive function. Importantly, xenoestrogens can also trigger non-genomic ER signaling *in vitro* through activation of PI3K/Akt, MAPK and PKA/PCK, albeit with tissue- and dose-specific effects on pathway activation (93, 95). The importance of appropriate ER-mediated signaling during development of the reproductive has been demonstrated in ER $\alpha$  knock-out mice (ERKO), which are resistant to the DES-induced alterations in gene expression and neoplasias of the uterus (270). Hence, inappropriate non-genomic ER activation during development by xenoestrogens could lead to permanent alterations in the uterine response to hormones.

Interestingly, non-genomic signaling can induce epigenetic modifications including cytosine and histone methylation. Several studies have demonstrated a role for epigenetic modulation by xenoestrogens, specifically DNA methylation perturbation. Alterations in DNA methylation have been observed after neonatal exposure to multiple xenoestrogens including DES, GEN and BPA for several

estrogen-responsive genes (i.e. *c-fos*, *lactoferrin* and *Nsbp1*) (170, 171, 174). The question of how epigenetic reprogramming of gene expression by xenoestrogens occurs, though, remains. In addition to DNA methylation, histone modifications can create binding sites for several enzymes, DNA and histone methyltransferases (HMT), that recognized site-specific chromatin marks. Specifically, histone methylation catalyzed by HMTs can result in the repression (i.e. H3K27) or activation (i.e. H3K4) of gene expression, in general. In our laboratory, we identified a mechanism by which DES can modulate non-genomic signaling through activation of the PI3K/Akt pathway leading to changes in phosphorylation of a histone methyltransferase, enhancer of zeste homologue 2 (EZH2). We demonstrated, *in vitro* and *in vivo*, that increased phosphorylation of EZH2 after DES exposure results in decreased levels of the target of EZH2 activity, tri-methylated lysine 27 on histone 3 (H3K27me3). Reduction of H3K27me3 levels, a repressive mark for gene expression, by siRNA knock-down of EZH2 correlated with the increased expression of developmentally reprogrammed genes previously identified by our laboratory, *PR* and *Igfbp5* (271). Because this mechanism of non-genomic ER signaling results in epigenetic modifications after DES exposure, the purpose of this study was to investigate whether this mechanism was shared by other xenoestrogens, GEN and BPA. Furthermore, like DES, we hypothesized that developmental reprogramming of gene expression by GEN and BPA would also modulate tumorigenesis in our animal model of uterine leiomyoma.

## **3.2 METHODOLOGY**

### **3.2.2 Animals and treatments**

Eker rats bred from a closed colony at The University of Texas M. D. Anderson Cancer Center in Smithville, TX, were cared for in accordance with the guidelines of the M. D. Anderson Cancer Center Animal Care and Use Committee in an ALAC accredited facility. Female Eker rats were maintained on standard rat chow (Harlan Teklad 22/5 rodent Diet), which is associated with a historical 65% tumor incidence in this animal model, and this was factored into baseline data. The rats were given water *ad libitum* and maintained on a 14:10 light-dark cycle. The reprogramming effects of xenoestrogens were examined after treating neonatal Eker rats on postnatal days 10 through 12 with BPA 50 mg/kg (Acros Organics, Morris Plains, NJ) or GEN 50 mg/kg

(Sigma Chemical Co., St. Louis, MO) dissolved (BPA) or suspended (GEN) in sesame oil, using a total of 50  $\mu$ l of this solution in vehicle for each subcutaneous injection. A separate group of animals were given vehicle (VEH) only as controls. Upon weaning, all animals were genotyped for the presence of the Eker mutation (*Tsc-2<sup>+/+</sup>* vs. *Tsc-2<sup>Ek/+</sup>*) and housed in no more than three per cage. For developmental reprogramming analysis, 61 animals were killed at 3 months by CO<sub>2</sub> asphyxiation, at which time reproductive tract morphology and gene expression were examined. Analysis of acute response to xenoestrogen was evaluated in 12 animals killed 6 hours after the last of 3 daily injections of xenoestrogen (postnatal day 12) by cervical dislocation. Female rats were killed at 16 months by CO<sub>2</sub> asphyxiation (VEH n=17, GEN n=14, BPA n=30) and ovaries, uteri and vagina were collected. Any gross tumors were also collected separately and snap frozen at -80°.

### **3.2.2 Tissue collection and histological studies**

Uteri from PND12, 5 month and 16 month were fixed in 10% neutral buffered formalin for 24-48 hours and then transferred into 70% ethanol before being embedded in paraffin and stained with hematoxylin-eosin (H&E) or incubated with antibody directed against Calbindin D9k (1:2000; Swant, Bellinoza, Switzerland) for 1hour as described previously (254). In addition to uteri, vagina and ovaries from 5 and 16 month old animals were also fixed, embedded and stained with H&E for estrus categorization. Tumors were measured and sectioned for pathological examination, and a portion of each snap-frozen in liquid nitrogen. Additionally, the uninvolved uterus was sectioned and analyzed for microscopic tumors, which together with quantitation of macroscopic lesions was used to calculate tumor incidence and multiplicity. Three-month-old animals were killed and their uteri were scraped with a sterile scalpel in cold PBS to remove endometrium from myometrium, were snap-frozen in liquid nitrogen and stored at -80°C. To obtain adequate amounts of tissue for RNA extraction from animals killed 6 hours after treatment on PND 12, two to three uteri were pooled together.

### **3.2.3 Histological categorization of estrous**

Reproductive staging was performed in accordance with the procedure described in chapter 2. Briefly, reproductive stage was determined by histological examination of the ovaries, vaginas, and uteri of 16- and 3-month-old rats. The 16-month-old rats were staged according to degree of reproductive senescence (pseudo pregnant, persistent estrus, or anestrus). 3-month-old rats were categorized as being in proestrus, estrus, metestrus, or diestrus stages of the estrus cycle.

### 3.3.4 Real-time PCR (qPCR)

As described in chapter 2, frozen tumors, myometrium or uteri from 16-month-old, 3-month-old and 12-day-old animals, respectively, were crushed under liquid nitrogen with mortar and pestle and RNA isolated and DNA removed by using the RiboPure™ Kit (Ambion Biosystems, Austin, TX) according to the manufacturer's protocol. Following RNA extraction, cDNA was made by reverse-transcribing 1 µg of RNA through the Invitrogen Superscript™ First-Strand Synthesis III System for reverse transcriptase (RT)-PCR (Invitrogen, Carlsbad, CA). Aliquots of cDNA were made for each sample and stored at -20°C until analyzed. cDNA from neonatal uteri, 3-month myometrium, or age- and stage-matched myometrium and tumors were subjected to quantitative real-time polymerase chain reaction analysis (qPCR). qPCR was performed by using the 7900T Fast Real-Time detection system from Applied Biosystems (ABI, Foster City, CA). Fast Real-Time Taq-Man assays from ABI were used exclusively to analyze expression of the *Gdf10* (Rn00666937\_m1), *Calbindin D9k* (Rn00560940\_m1), *Rasd2* (Rn00592054\_m1), *Sfrp2* (Rn01458836\_m1), *Krt19* (Rn01496867\_m1), *Nr2f2* (Rn00756178\_m1), *Gria2* (Rn00568514\_m1), *Igfbp5* (Rn00563116\_m1), *Spp1* (Rn00563571\_m1), *Car8* (Rn01473820\_m1), *Mmp3* (Rn00591740\_m1), *Tacst1* (Rn00684677\_m1), *Rps6k* (Rn00667685\_m1), *Kcnk2* (Rn00572452\_m1), *Cspg2* (Rn01493763\_m1), *Aqp3* (Rn00581754\_m1), *Ramp1* (Rn00671666\_m1) and *Dio2* (Rn00581867\_m1) genes. All Q-PCR reactions were performed by mixing Universal Fast Real-Time Master Mix from ABI together with the gene assay mix first and then adding 2 µl of cDNA from each sample to make up a 25-µl volume. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) or ribosomal 18s (*18s*) were used as endogenous controls, including probe and forward and reverse primers in a 25-µl reaction volume. Specifically, *18s* was utilized for comparison to

estrogen-responsive genes in the adults due to significant association of *GAPDH* expression with BPA exposure. The following set of conditions were used for each fast Q-PCR reaction: 95°C for 10 minutes, followed by 40 cycles of 1 second at 95°C and 20 seconds at 60°C.

The qPCR reactions were all performed in triplicate and were quantified by the  $-\Delta\Delta C_T$  method, which uses the average  $C_T$  of the *GAPDH* or *18s* subtracted from the target gene  $C_T$  to obtain the average  $\Delta C_T$ . A calibrator was chosen from each set of samples from which to subtract individual GEN, and BPA sample  $\Delta C_T$  values to obtain  $-\Delta\Delta C_T$ . The fold change for each sample was calculated in comparison to the calibrator by taking  $2^{-\Delta\Delta C_T}$ . The calibrator for both 16-month-old and 12-day-old rats was VEH-treated myometrium or uteri. For the 3-month-old rats, an estrus-staged matched VEH myometrium was used.

### **3.2.5 Preparation of tissue lysates and immunoblotting**

Frozen tissue from neonatal animals (PND 12) treated with VEH, GEN or BPA and harvested at 1, 6 or 12 hrs after the injection was used for the preparation of protein lysates for immunoblotting. Uteri from 3 neonatal animals were pooled together in RSB buffer (10 mM Tris HCl pH7-7.4, 10 mM NaCl, 3 mM MgCl<sub>2</sub>, 1 mM PMSF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, and Complete Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN)) with 0.5 % NP-40. Tissue was homogenized using a dounce homogenizer and incubated on ice for 10 min. Tissue lysates were then centrifuged for 15 min at 15,000 rpm at 4°C and supernatant collected. Protein concentration of tissue lysate was determined using the Pierce BCA assay (Thermo Fischer Scientific, Rockford, IL). 10-20 µg of tissue lysate was separated via SDS-PAGE on 10% gels (BioRad Laboratories, Hercules, CA) and transferred overnight at 4°C to polyvinylidene difluoride (PVDF) membranes that were activate with methanol. Membranes were blocked in 1X tris-buffered saline plus 0.5% Tween 20 (TBST) and 5% non-fat milk for 1 hour. Membranes were washed briefly with 1 X TBST and then incubated with primary antibody for 2hrs or overnight at 4° and washed with TBST followed by incubation for 1-2hrs with HRP-conjugated secondary antibody.

Visualization of protein abundance was performed using Pierce ECL substrate or ECL plus (Thermo Fischer Scientific, Rockford, IL) on autoradiograph film (Biomax MR, Kodak, New York, NY). Immunoblotting was performed with antibodies against phosphorylated Akt (S473) and (T308), Akt, phosphorylated S6 (S235/236), S6, and histone H3 obtained from Cell signaling Technology (Montgomery, TX). Antibodies against H3K27me3 and EZH2 were obtained from Active Motif (Carlsbad, CA). The antibody recognizing phosphorylated EZH2 (S21) was obtained from Bethyl Incorporated (Montgomery, TX).

### **3.2.6 Immunohistochemistry**

Tissue embedded in paraffin were cut into 5  $\mu$ m sections and placed on slides for deparaffinization. Slides were heated at 60°C for 1hr and then in further deparaffinized in Citrusolv for 5 minutes 3 times. This was followed by rinsing in 100% EtOH for 2 minutes twice. Sections were progressively rehydrated in 90%, 80% and 70% EtOH for 2 minutes each and rinsed with water for 1minute. Slides were then boiled in Antigen unmasking solution for 5 minutes and allowed to cool for 30 minutes, which were then rinsed with PBS for 5 minutes twice. Blocking of endogenous peroxidase activity was performed in 2% H<sub>2</sub>O<sub>2</sub> for 30 minutes and rinsed in PBS. After washing in PBS, non-specific binding with blocked with Avidin D and Biotin for 15 minutes and washed with PBS. Sections were then incubated overnight at 4° in primary antibody, calbindin D<sub>9</sub>K (1:2000, Swant), in 5% goat serum plus 0.3% Triton-X, and then rinsed in 0.03% TBST for 10 minutes twice and washed with PBS for 10 minutes. Sections were then incubated with horseradish peroxidase-conjugated secondary antibody for 1hr and visualized by staining with Tablet DAB (Sigma Chemical Company).

### **3.2.7 Immunoprecipitation**

Tissue lysates from the above preparation were pre-cleared using protein A sepharose beads (GE healthcare, Piscataway, NJ) and rabbit IgG (Millipore, Billerica, MA). Pre-cleared lysates were incubated with antibody against phosphorylated EZH2

and protein A sepharose beads and then washed with Cell Signaling Technology (CST) lysis buffer (20 mM Tris-HCl pH 7.5, 1 mM EGTA, 1 mM Na<sub>2</sub>EDTA, 150 mM NaCl, 1 mM beta-glycerophosphate, 2.5 mM sodium pyrophosphate, 1% Triton-X, 1 mM PMSF, 1 mM NaF and Roche Complete Protease Inhibitor Cocktail). 100-500 µg of protein lysate from immunoprecipitation was separated and immunoblotted as describe previously.

### **3.2.8 Acid Precipitation of Histones from Tissue Lysate**

Cell pellets obtained from preparation of tissue lysates describe above, were resuspended in a 1:1 ratio of 5 mM MgCl<sub>2</sub> and 0.8 M HCl and sonicated for 20 sec (30% power) followed by a 1hr incubation on ice. Histone proteins were collected by centrifugation for 10 min at 14,000 rpm (4° C), supernatant transferred to a new tube and precipitated with trichloroacetic acid (50%) and ddH<sub>2</sub>O. Histone precipitants were collected after centrifugation for 20 min at 14,000 rpm (4° C). Histone pellets were washed with cold acetone and allowed to dry before reconstituting in a solution of 1.5 M Tris-HCl pH 8.8 and ddH<sub>2</sub>O. Histones were quantitated after separation on Tris-tricine gels (10-20%) followed by staining with coomassie. After estimating protein abundance histones were loaded equally and SDS-PAGE was performed using 10-20% Tris-tricine gels, followed by transferring to PVDF. Immunoblotting was performed as describe above with total H3 used to determine relative histone methylation levels or with antibody against H3K27me3.

### **3.2.9 Statistics**

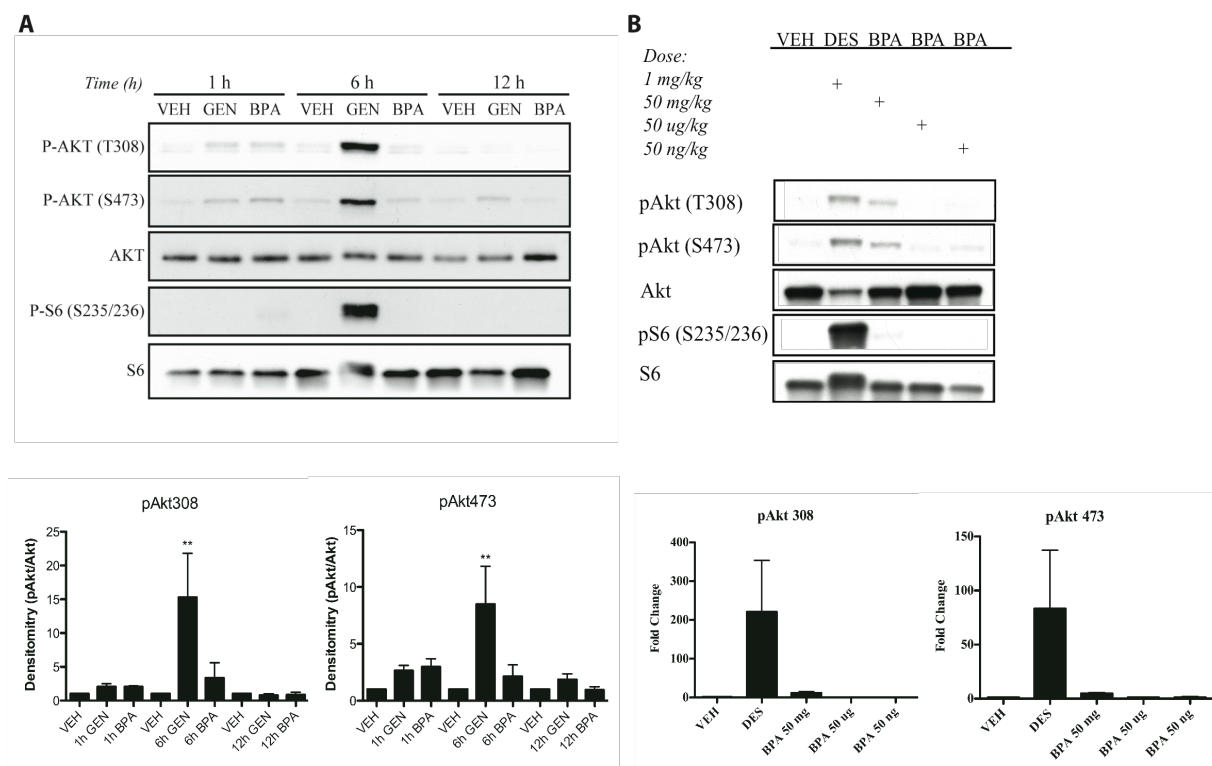
For statistical analysis of qPCR data, a linear model analysis was applied to  $\Delta C_T$  values to determine the effects of gene and treatment, which were determined to be significant at  $p$  value of  $<0.05$ . Analysis of tumor incidence was estimated using chi-square for the determination of significance between treatment groups. Tumor multiplicity was analyzed using the Poisson regression for comparing multiple tumors between VEH, GEN and BPA groups. A value of  $p < 0.05$  was considered statistically

significant. For detecting quantitative differences in immunoblotting experiments, a Students t-test was applied to densitometry values (n=3) to determine significance.

### **3.3 RESULTS**

#### **3.3.1 Xenoestrogen-specific activation of non-genomic signaling in the neonatal uterus**

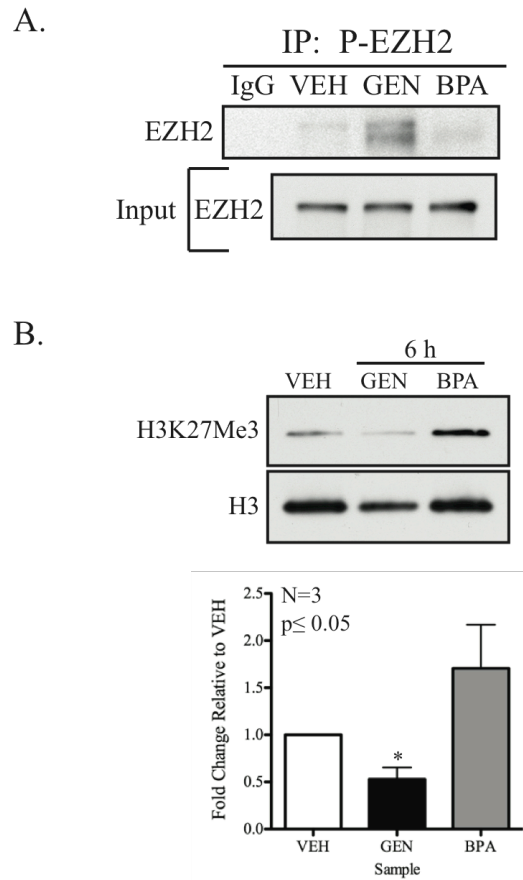
In our previous report, (271) we established that neonatal DES exposure initiates rapid ER-dependent activation of the PI3K/Akt pathway, which also induced phosphorylation of S6, the downstream readout of mTOR activation. In this study we investigated the ability of GEN and BPA to induce rapid activation of Akt and S6 after neonatal exposure to DES (1 mg/kg), GEN (50 mg/kg), three different doses of BPA (50 mg/kg, 50 µg/kg or 50 ng/kg) or VEH over a time course of 1, 6 and 12 hrs after subcutaneous injection. On PND 12, a period defined as susceptible to developmental reprogramming by DES (254), uteri exposed to GEN or DES (control) demonstrated activation of Akt and S6 at 6hrs as illustrated in **Figure 3.1A** by the significant increase in phosphorylation at S473 and T308 on Akt and S235/236 on S6. In contrast, neither BPA, at any dose, or VEH induced significant activation of this pathway at any of the time points tested (**Figure 3.1B**). Using DES as a control, these data support our previous *in vitro* and *in vivo* findings showing activation of non-genomic ER signaling via Akt, but also demonstrate *in vivo* that xenoestrogens do not share the same mechanism of activation in the developing uterus.



**Figure 3.1 Xenoestrogen-specific modulation of non-genomic PI3K/AKT signaling.** A) Western blot analysis of AKT phosphorylation (T308 and S473) and S6 phosphorylation in pooled (3) uteri of PND 12 Eker rats after exposure for 1-12 hrs to VEH, GEN (50 mg/kg) or BPA (50mg/kg) via s.c. injection. B) Lysates were collected from pooled (2-3) Eker rat uteri on PND 12 exposed to 50 mg/kg, 50 µg/kg or 50 ng/kg of BPA or VEH for 6hrs. Western blot analysis and densitometry of uteri revealed no significant activation of the PI3K/AKT pathway as compared to VEH. Densitometric quantification of western blot analysis demonstrates significant differences in activation of AKT as represented by the ratio phospho-AKT T308 and S473 to total AKT in xenoestrogen-exposed animals vs. VEH. Mean  $\pm$  SEM is donated by error bars on each graph. The Student's t-test was used to determine statistical significance, which was set at a value of  $*p < 0.05$ .

### 3.3.2 Xenoestrogen-specific modulation of EZH2 in the neonatal uterus

As EZH2 is critical to embryonic development, it is important to investigate the impact inappropriate exposure to xenoestrogens has on its activity and expression. In a similar manner to DES, GEN exposure also results in increased phosphorylation of EZH2 at time frame that corresponds to the activation of Akt (**Figure 3.2**). However, this phosphorylation event was not observed in BPA or VEH exposed uteri (**Figure 3.2**). Importantly, the target of EZH2 activity H3K27me3 was reduced in GEN exposed animals, as shown in **Figure 3.2** by decreased levels of H3K27me3 in chromatin. At 6hrs after exposure to BPA this trend was not observed, which is consistent with lack of activation of Akt and phosphorylation of EZH2 (**Figure 3.2**). Together this evidence supports that rapid activation of Akt via ER in the developing uterus has xenoestrogen-specific effects on EZH2 activity, which modulates global H3K27me3 levels in chromatin.



**Figure 3.2 Xenoestrogen-specific modulation of EZH2 and H3K27me3.** Neonatal PND 12 pooled (2-3) uteri from Eker rats were homogenized 6hrs after exposure to VEH, GEN (50 mg/kg) or BPA (50 mg/kg) and immunoprecipitated with anti-phospho-EZH2 antibody. Western blot analysis of EZH2 from immunoprecipitants demonstrate a significant enrichment of phospho-EZH2 in uteri from GEN-exposed rats as compared to BPA and VEH. PND 12 pooled (2-3) from Eker rat uteri exposed to VEH, GEN or BPA (6 hrs) were used for acid precipitation of histones. Western blot analysis of histone proteins show a decrease in levels of H3K27me3 at 6hrs after exposure as compared to BPA or VEH. The ratio of phospho-EZH2 to total EZH2 and the ratio of H3K27me3 to total H3 levels were determined to be significantly different from VEH using the Student's t-test, which was set a value of \* $p < 0.05$ .

### 3.3.3 Neonatal exposure to GEN and BPA induces precocious expression of estrogen-responsive genes

It is known that epigenetic changes occur rapidly during development, and with respect to DNA methylation, it has been shown that exposure to DES and GEN can transiently alter DNA methylation patterns in the uterus that are permanently reversed when endogenous hormones are produced. These changes in DNA methylation also correlate with persistent alterations in gene expression in the adult. We previously identified a set of 18 estrogen-responsive genes, from a microarray-based analysis that were candidates for developmental reprogramming in the uterine myometrium (269). To determine a baseline of gene expression in the developing uterus after neonatal exposure to GEN and BPA, we isolated mRNA from neonatal uteri (VEH n=9, GEN n=6, BPA n=6) and performed qPCR to quantitate expression of this panel of 18 genes. Twelve of the 18 target genes were responsive to one or both xenoestrogens, being either induced or repressed by GEN and/or BPA compared to VEH controls. GEN modulated the expression of all 12 genes (*Calbindin D9k*, *Dio2*, *Krt19*, *Gdf10*, *Car8*, *Gria2*, *Mmp3*, *Igfbp5*, *Spp1*, *Sfrp2*, *Rasd2*, *Nr2f2*) (**Table 3.1**). In comparison, BPA modulated the expression of eight of the 12 genes (*Calbindin D9k*, *Dio2*, *Gdf10*, *Car8*, *Gria2*, *Spp1*, *Sfrp2*, *Rasd2*) (**Table 3.1**). While qualitatively similar in terms of induction or repression of expression, the response of these estrogen-responsive genes to both GEN and BPA was quantitatively different with GEN resulting in the induction of 12/12 genes and, in general, a greater increase in expression as compared to BPA. These data indicate that while GEN and BPA are qualitatively similar, they have xenoestrogen-specific effects in their ability to modulate estrogen-responsive gene expression in the developing rat uterus.

**Table 3.1.** Neonatal uterine gene expression in response to xenoestrogen exposure.

Gene	Normal	Neonatal DES Response <sup>a</sup>	Neonatal GEN Response	Neonatal BPA Response
	Estrogen Response			
		Fold change±SEM	Fold change±SEM	Fold change±SEM
<i>Calbindin D9k</i>	Induced	39.12±1.38	52.30±1.96	28.10±1.24
<i>Dio2</i>	Induced	7.31±0.28	6.63±0.21	9.51±0.6
<i>Krt19</i>	Induced		2.05±0.13	NSD
<i>Gdf10</i>	Repressed	-4.78±0.06	-4.78±0.08	-5.00±0.06
<i>Car8</i>	Repressed	-7.14±0.05	-20.1±0.05	-12.5±0.04
<i>Gria2</i>	Repressed	-83.3±0.03	-33.1±0.07	-11.1±0.01
<i>Mmp3</i>	Repressed	-3.85±0.08	-2.50±0.05	NSD
<i>Igfbp5</i>	Repressed		-3.53±0.09	NSD
<i>Spp1</i>	Repressed		-2.97±0.13	-3.58±0.07
<i>Sfrp2</i>	Repressed		-9.13±0.08	-4.35±0.13
<i>Rasd2</i>	Repressed		-5.28±0.17	-2.53±0.14
<i>Nr2f2</i>	Repressed		-4.63±0.19	NSD

<sup>a</sup>Data previously reported in (Greathouse et al. 2008)

Fold change relative to vehicle, SEM; standard error of the mean.

Values statistically significant at p<0.05 using one-way ANOVA.

NSD: No significant difference

**TABLE 3.1****Table 3.2.** Adult myometrial gene expression in response to GEN or BPA exposure.

Gene	Normal	Adult GEN Response	Adult BPA Response
	Estrogen Response		
		Fold change±SEM	Fold change±SEM
<i>Calbindin D9k</i>	Induced	3.8±0.15 <sup>#</sup>	-3.30±0.21 <sup>*</sup>
<i>Dio2</i>	Induced	-2.1±0.09 <sup>#</sup>	NSD
<i>Krt19</i>	Induced	2.3±0.13 <sup>#</sup>	NSD
<i>Gdf10</i>	Repressed	2.9±0.19 <sup>#</sup>	-2.8±0.21 <sup>*</sup>
<i>Gria2</i>	Repressed	4.4±0.16 <sup>#</sup>	-2.8±0.33 <sup>*</sup>
<i>Igfbp5</i>	Repressed	-13.9±0.12 <sup>#</sup>	NSD
<i>Spp1</i>	Repressed	-2.2±0.13 <sup>#</sup>	NSD
<i>Sfrp2</i>	Repressed	NSD	-2.0±0.19 <sup>*</sup>
<i>Rasd2</i>	Repressed	NSD	6.3±0.28 <sup>*</sup>

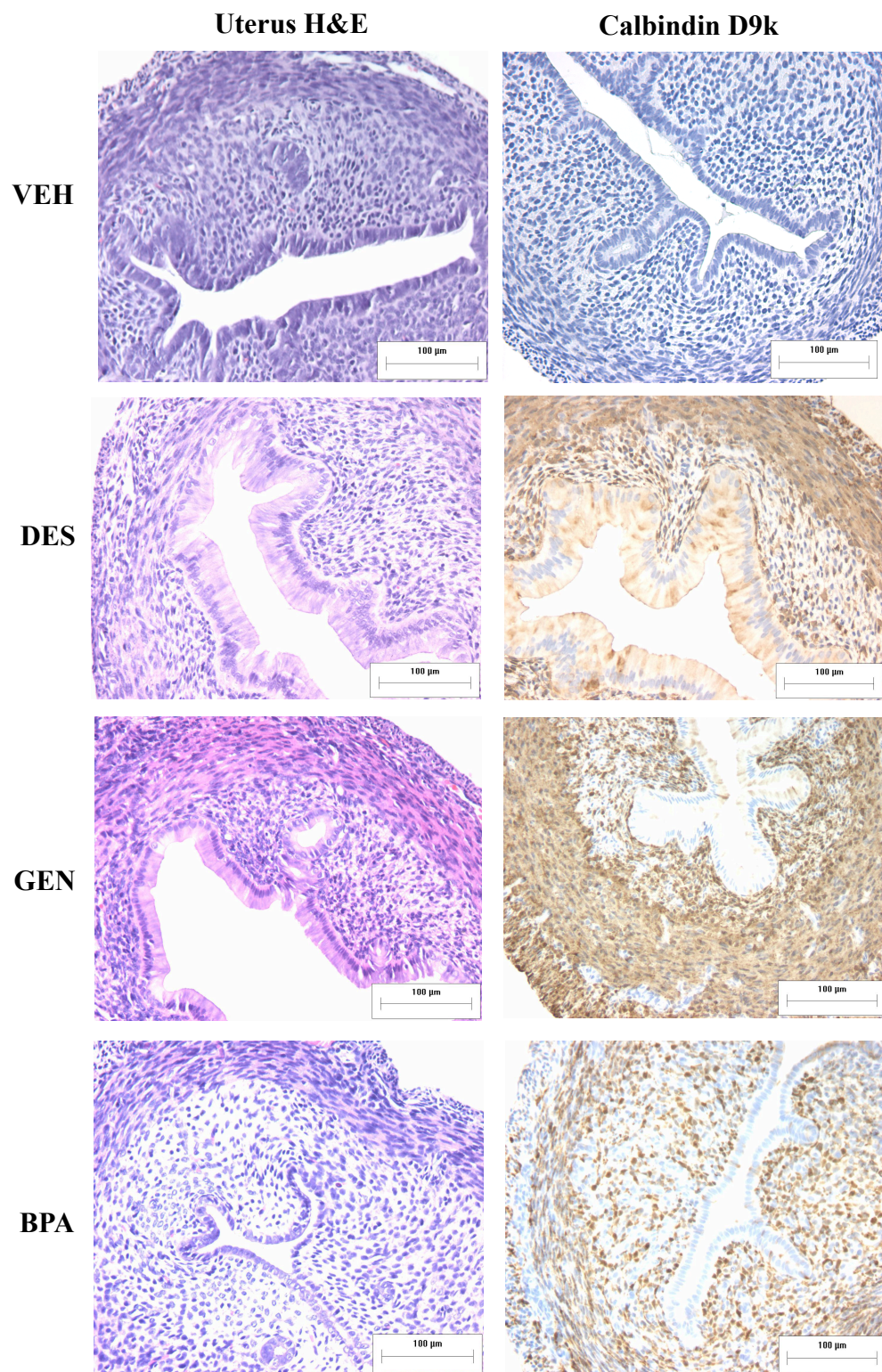
Fold change relative to vehicle, SEM; standard error of the mean.

Values statistically significant at <sup>\*</sup>p<0.01 and <sup>#</sup>p<0.05 using one-way ANOVA.

NSD: No significant difference

**TABLE 3.2**

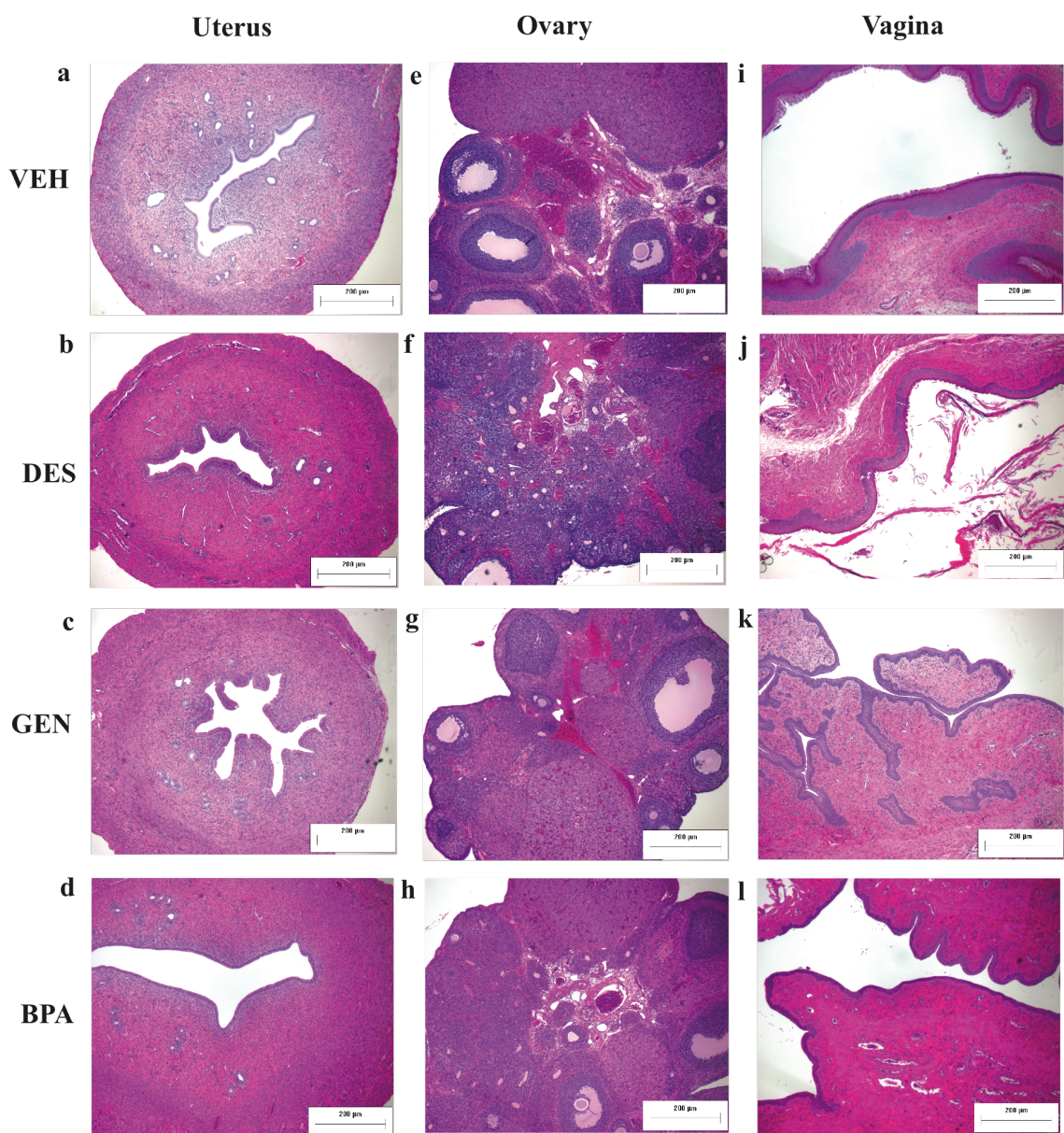
Transactivation of gene expression was confirmed at the protein level by examining calbindin D9k expression in response to xenoestrogens. Neonatal uteri from animals exposed to GEN or BPA exhibited induction of the classic uterine estrogen-responsive gene *Calbindin D9k* by immunohistochemistry (IHC) as shown in **Figure 3.3**, confirming that these doses of GEN and BPA are estrogenic in the neonatal rat uterus, which has been previously shown to induce *Calbindin D9k* expression in neonatal rats relative to VEH controls (254).



**Figure 3.3 Histological and immunohistochemical studies of uteri of 12-day-old Eker rat females.** Photomicrographs of uteri stained with either hematoxylin and eosin or calbindin D9K in VEH-, DES-, GEN-, or BPA-exposed females killed 6 hours after the last exposure on PND 12 (all photomicrographs were taken at 20X). The length of the scale bar is 100  $\mu$ m.

### **3.3.4 Neonatal xenoestrogen exposure developmentally reprograms gene expression in adult myometrium in the absence of morphological reprogramming**

DES has been shown to reprogram female rodent reproductive tract morphology by inducing persistent vaginal cornification (persistent estrus), endometrial hyperplasia, and polycystic ovaries lacking corpora lutea in animals exposed neonatally to this xenoestrogen (254). IHC evaluation of the uteri, ovaries, and vaginas from adult female rats exposed neonatally to BPA (n=17), GEN (n=14), or VEH (n=34) demonstrated that the effect of these xenoestrogens on reproductive tract morphology differed substantially from DES (**Figure 3.4**). Adult females exposed neonatally to BPA or GEN had a more normal reproductive tract morphology as illustrated by the presence of corpora lutea in the ovary, normal endometrium luminal epithelium cell height, and normal estrus cycles (**Figure 3.4 g-l**).



**Figure 3.4 Morphological reprogramming of the adult Eker rat uterus.** Effects of neonatal exposure to DES, GEN, or BPA on adult uterine morphology. Shown are photomicrographs of hematoxylin and eosin–stained histological sections of uteri, ovaries, and vaginas from adult (3-5 months old) rats exposed to VEH (a, b and c), DES(d, e and f), GEN (g, h and i), or BPA (j, k and l) (20X). The length of scale bar is 200 µm.

After establishing the immediate effects of xenoestrogen exposure on gene expression, we next asked whether the xenoestrogen-specific non-genomic signaling effects of neonatal GEN and BPA exposure had induced persistent developmental reprogramming of gene expression in the adult myometrium. For this analysis, we defined a developmentally reprogrammed gene as one that displays altered hormone responsiveness in adult rat myometrium as a result of developmental xenoestrogen exposure. To control for differences in hormone levels associated with different stages of the estrus cycle, 3-month-old VEH-, GEN- or BPA-exposed female rats were grouped into proliferative phase, corresponding to animals in proestrus or estrus, or secretory phase, corresponding to animals in metestrus and diestrus. For the qPCR determination of levels of gene expression, average fold change in expression compared to VEH controls was normalized to the reference gene *18s*.

Interestingly, developmental reprogramming by GEN and BPA occurred in genes reprogrammed by DES, as well as in gene sets specific for each xenoestrogen. As shown in Table 3.1, of the 18 uterine estrogen-responsive genes previously identified (269), three were developmentally reprogrammed by all three xenoestrogens: *CalbindinD9k*, *Gdf10* and *Gria2*. However, while both DES and GEN reprogrammed these genes in such a way as to significantly increase expression by 2-4-fold during the proliferative phase of the estrus cycle (when hormone levels are highest), BPA had the opposite effect on these genes, with reprogramming by this xenoestrogen resulting in a 3-fold decrease in expression during the proliferative phase of the estrus cycle.

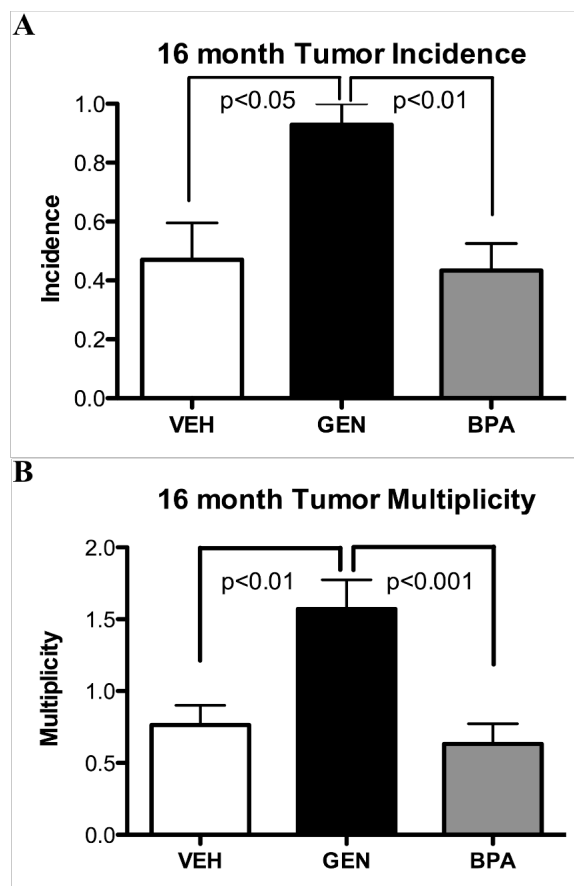
In addition to genes that were targeted by all three xenoestrogens, GEN and BPA also had xenoestrogen-specific reprogramming effects. As shown in Table 3.2, neonatal GEN exposure reprogrammed expression of *Dio2*, *Krt19*, *Igf1bp5* and *Spp1*, only one of which, *Dio2*, was also reprogrammed by DES (269). In contrast, BPA exposure resulted in developmental reprogramming of *Sfrp2* and *Rasd2* neither of which were reprogrammed by either DES or GEN.

While all 11 genes reprogrammed by DES, GEN and/or BPA were responsive to these xenoestrogens in the neonate, not all were developmentally reprogrammed in the adult myometrium. 4/7 genes became hyper-responsive to estrogen by GEN exposure, while 4/5 genes became further repressed by BPA exposure. Thus, a genomic response to xenoestrogen was insufficient to predict developmental

reprogramming or its xenoestrogen-specific effects. This indicates that the non-genomic effects observed with GEN exposure, and previously with DES, together with changes in EZH2 activity and levels of H3K27me3 were likely initiating mechanisms responsible for the differential reprogramming effects observed in adult myometrium. Thus, the genomic response following xenoestrogen exposure (i.e. induction or repression of gene expression) masked the xenoestrogen-specific developmental reprogramming effects until the promotional effects of the adult hormonal milieu caused it to be revealed.

### **3.3.5 Genistein but not BPA exposure increases susceptibility to uterine tumors**

The distinct non-genomic signaling and reprogramming profile of GEN and BPA led us to determine the impact of neonatal exposure to these xenoestrogens on susceptibility to develop uterine leiomyoma in Eker rats. Tumor incidence and multiplicity was determined at 16 months, the age associated with a historical tumor incidence of 65% (272), in animals exposed on neonatal days 10-12 to GEN, BPA or VEH. GEN exposure increased tumor incidence to 93% (GEN [n=14] vs. VEH [n=17],  $p < 0.05$ ), a significant increase above baseline tumor formation in VEH animals (**Figure 3.5a**). Tumor multiplicity was also increased by GEN (1.6) exposure vs. VEH controls (0.6) (**Figure 3.5b**). The increase in tumor incidence and multiplicity with neonatal GEN exposure was comparable to DES exposed animals, which exhibited a 100% tumor incidence and multiplicity of 1.3 (254). In contrast to both DES and GEN, BPA did not significantly increase tumor incidence or multiplicity in comparison to VEH controls, differing significantly from the effects of GEN on tumor formation (GEN vs BPA [n=30],  $p < 0.01$ ) (**Figure 3.5**). Tumor multiplicity followed a similar pattern in GEN-, BPA-, and VEH-exposed animals. These data indicate that the neonatal effects of GEN induced immediately by exposure and established by endogenous estrogen created an environment more susceptible to the effects of loss of *Tsc2* leading to increased tumor development.



**Figure 3.5 Tumor incidence and multiplicity of Eker rats exposed neonatally to GEN, BPA or VEH.** Incidence and multiplicity of uterine leiomyomas in Eker rats after xenoestrogen exposure. A) Tumor incidence in 16 month old Eker rats (gross and microscopic tumors) after GEN (n=14), BPA (n=30) or VEH (n=17) exposure. B) Tumor multiplicity in 16 month old Eker rats (gross and microscopic tumors). Statistical significance for incidences was determined by chi square for incidence and by Poisson regression for multiplicity analysis.

### 3.4 DISSCUSSION

In our previous study, we identified a new mechanism of ER-mediated non-genomic signaling that activates the PI3K/Akt signaling pathway to modulate the phosphorylation of the HMT, EZH2 and gene expression. Using xenoestrogens as a biological tool to identify genes that control reproductive tract differentiation and susceptibility to hormone-dependent tumors in adults, we reveal in this study that xenoestrogens have distinct non-genomic signaling profiles and chromatin effects that correlate with developmental reprogramming of estrogen-responsive genes. Rapid ER activation of Akt, which repressed the activity of EZH2 and levels of H3K27me3, distinguished GEN from BPA immediately after exposure. This distinction persisted as evidenced by the opposite effects GEN and BPA had on developmental reprogramming of gene expression in the adult uterus, as well as, the modulation of tumorigenesis.

The mechanism by which aberrant hormone exposure induces changes in chromatin structure is poorly understood. Changes in histone methyl marks, as well as cytosine methylation, are known to be heritable (273), and have been shown to modulate one another through several mechanisms (163, 274-276). Regulation of H3K27 methylation levels by EZH2 is required for several key functions in development including X inactivation, bivalent chromatin maintenance and silencing of *HOX* genes, which have been shown to be critical to proper murine uterine differentiation. In a recent study of the methylome at various stages during differentiation, the most significantly hypomethylated regions in embryonic stem cells (hESC) were located in the *HOX* genes, with a progressive increase in methylation associated with differentiation state (277). Interestingly, in Fibro cells representing neonatal differentiation, *HOXC* and *HOXD* showed maximal DNA methylation, which decreased in the fully differentiated cells (monocytes) indicating that these gene clusters undergo key epigenetic modifications during later stages of differentiation. Additionally, EZH2 can directly mediate gene silencing of estrogen-responsive genes by inducing DNA methylation via interactions with the histone deacetylase HDAC1 followed by recruitment of DNA methyltransferases. Therefore, the reduction in levels of H3K27me3 by xenoestrogen exposure, which may reduce levels of DNA methylation in developmentally regulated estrogen-responsive genes, could give rise

to aberrant differentiation of the uterus. The epigenetic heritability of these methyl marks indicates that developmental reprogramming of the epigenome could lead to permanent changes in genes expression that increase susceptibility to hormone-dependent tumors.

The mechanism of epigenetic alteration after neonatal xenoestrogen exposure has been shown to lead to persistent changes in gene expression that are associated with neoplastic transformation. The precocious response to xenoestrogens seen immediately after exposure showing during critical windows of uterine development (i.e. PND 1-5) permanently alters normal estrogen-responsive gene expression, such as *lactoferrin* and *c-fos*, via DNA hypomethylation that persists in tumors (i.e. uterine adenocarcinomas) (170, 222, 223). In our study, genes that became developmentally reprogrammed responded to xenoestrogens, being precociously activated or repressed immediately after xenoestrogen exposure in neonates (postnatal day 12) in a pattern reflective of their normal response to hormone in the adult. For example, both GEN and BPA induced activation of *Calbindin D9k* in the neonatal uterus, which mimicked the normal hormone-responsive behavior of this gene during the proliferative phase of the estrus cycle in the adult myometrium. In contrast to the response of target genes to xenoestrogen in estrogen-naïve neonatal uteri, which mimicked the normal response to endogenous hormone, the effects of reprogramming of hormone responsiveness later in life in the adult uterus differed both quantitatively (i.e. 2-50 fold increased expression) and in many cases qualitatively (i.e. repressed genes became induced) relative to normal response of these genes to hormone in the adult myometrium. The ability of neonatal xenoestrogen exposure to “reset” the normal hormone responsiveness has also been demonstrated after brief exposure to DES or BSA conjugated to E2, which allows only the non-genomic signaling of E2 to be investigated. In our study, we demonstrated that a 7-day estrogen exposure (DES or E2-BSA) and 48 h recovery followed by estrogen challenge reprograms the normal estrogen-responsiveness to estrogen in myometrial cells. Genes identified in this study and in previous studies as developmentally reprogrammed by xenoestrogen exposure, *PR* and *Igfbp5*, become hyper-responsive to hormone, mimicking what occurs in adult myometrium after neonatal exposure to xenoestrogens. Similarly, inappropriate glucocorticoid exposure remodels chromatin around the glucocorticoid receptor (GR)-regulated tyrosine amino transferase gene (*Tat*) and induces DNA demethylation at

GR-response enhancer regions, which recruits transcription factors and results in gene activation (189). Long after removal of glucocorticoids, however, the alterations in DNA demethylation persisted giving rise to a hyper-responsiveness to glucocorticoids when later exposure occurred resulting in increased *Tat* gene expression. While the mechanism of hormone-dependent DNA demethylation is not well understood, in a recent study it has been demonstrated that exposure to PTH engages the non-genomic signaling pathway via PKC, which leads to phosphorylation of methyl binding protein 4 (MBD4) (278). MBD4 recruits DNA base excision repair machinery, which results in demethylation of the vitamin D receptor-dependent promoter of the cytochrome p450 27B1 (CYP27B1) gene. In addition to the mechanism we have illustrated of DES-induced activation of Akt and phosphorylation of EZH2, these data provide additional evidence that chromatin remodeling can be permanently altered after inappropriate hormone exposure via non-genomic signaling pathways leading to a persistent hypomethylated state and permanent alterations in gene expression.

Overall, the xenoestrogen-specific patterns of gene expression that emerged from this analysis reveal that the intrinsic reprogramming effects associated with specific xenoestrogens are different. GEN developmentally reprogrammed, *Gdf10*, *Calbindin D9k*, *Gria2*, *Dio2*, *Krt19*, *Igfbp5*, and *Spp1*; the first five of which were also reprogrammed by DES (269). In contrast, while BPA reprogrammed *Gdf10*, *Calbindin D9k*, *Gria2*, *Rasd2*, and *Sfrp2*, unlike DES and GEN, with the exception of *Rasd2*, BPA reprogramming resulted in decreased gene expression. Therefore, in general, the effect of BPA on gene expression was the opposite of DES and GEN. These findings are reminiscent of those reported by Dolinoy et al. (279), where developmental exposure to GEN and BPA had opposite effects on DNA methylation and subsequent gene expression of the agouti gene. Together with our previous report (269) these data indicate that xenoestrogens such as DES, BPA or GEN have both shared and distinct effects on gene expression and developmental reprogramming of target genes.

Other differences between the effects of these xenoestrogens on the developing uterus were also observed. Morphological reprogramming of the reproductive tract following neonatal GEN and BPA exposure was not observed, unlike what was seen in this model with DES (254). However, other studies have reported reproductive abnormalities as a result of perinatal exposure to GEN and BPA (51, 53, 64, 72, 75, 280-282). Several possibilities exist for these discordant observations

including differences in dose, route of administration, timing of administration, background strain, and species, though all three doses of BPA did not result in non-genomic activation of Akt, in contrast to DES and GEN. Regardless, data obtained in this rat model system clearly demonstrate that reprogramming of tumor susceptibility and estrogen-responsive gene expression at the molecular level can occur at doses of xenoestrogens that do not cause overt morphological changes in the female reproductive tract. Similar to our observations, Adachi et al. demonstrated that molecular alterations in the testis can occur in the absence of morphological alterations (283). In this study, while neonatal DES exposure induced both morphological and molecular alterations in the testis, GEN reprogrammed genes in the adult testes without inducing morphological reprogramming. Therefore, the effects of developmental reprogramming may be manifest in the absence of histological or morphological alterations, pointing to the need to develop approaches, like that of profiling non-genomic signaling and epigenetic alterations, to detect reprogramming even in morphologically “normal” appearing tissues.

Futhermore, we found that the pattern of xenoestrogen-induced developmental reprogramming in genes targeted by all three xenoestrogens examined (GEN, BPA and DES), correlated with the ability of these xenoestrogens to increase susceptibility to tumor formation. DES and GEN generally increased the expression of estrogen-responsive genes in adult animals relative to VEH-exposed rats, which mimicked the significant increase in tumor formation in DES and GEN exposed animals. In contrast, BPA resulted in general repression of estrogen responsive genes, and no increase in tumor formation and multiplicity as compared to VEH. These differences in the effects of xenoestrogen exposure are likely driven by intrinsic differences in the mechanism of action of each of these xenoestrogens, for example binding to specific ER subtypes. DES, GEN and BPA have distinct binding efficiencies for ER $\alpha$  and ER $\beta$ , with DES and GEN binding with a higher affinity to ER $\alpha$  than BPA (DES>GEN>BPA), while BPA binds with a much higher affinity to ER $\beta$  than ER $\alpha$  (15). Additionally, BPA, in contrast to DES, can engage the G-coupled receptor pathway to activate non-genomic signaling through PKA resulting in phosphorylation of CREB and Rb *in vitro* (105). However, ablation of ER via ICI 182780 treatment did not abrogate the effects of BPA illustrating that this is an ER-independent mechanisms of BPA. Additionally, neither E2

nor DES could recapitulate the BPA-induced effects further illustrating the unique effects of BPA.

In the female reproductive tract, it has been demonstrated that ER $\alpha$  is required for DES-induced developmental reprogramming, as  $\alpha$ ERKO mice are resistant to DES-induced developmental reprogramming (35, 193) and overexpression of ER $\alpha$  increases the effects of DES (284). Importantly, in our previous study we demonstrated that ERKO mice are resistant to the non-genomic effects of DES exposure, which would support the hypothesis that early epigenetic signaling events are ER-mediated. Similarly, xenoestrogen-specific affinity for ER $\alpha$  vs ER $\beta$  has been demonstrated in male  $\alpha$ ERKO and  $\beta$ ERKO mice exposed neonatally to DES (285). This study demonstrated that while DES exposure induced prostate abnormalities in the  $\beta$ ERKO mice, that expressed ER $\alpha$ ,  $\alpha$ ERKO mice that lack this receptor were resistant to the effect of DES exposure. In this regard, the ability of DES and GEN to reprogram susceptibility to tumorigenesis is consistent with ER $\alpha$  being the predominate ER subtype in the female reproductive tract (286, 287).

In summary, this study demonstrates that neonatal exposure to GEN, in contrast to BPA, activates non-genomic signaling to Akt, which phosphorylates EZH2 and reduces H3K27me3 levels in the neonatal uterus. This disparate activity of GEN and BPA persisted in the adult myometrium as evidenced by the opposing effects on developmental reprogramming of estrogen-responsive genes and subsequently on tumorigenesis. The findings of the present study demonstrate a correlation between tumor incidence and xenoestrogen-specific reprogramming of estrogen-responsive genes following neonatal chromatin remodeling events, and highlight what are likely important intrinsic differences between xenoestrogens. While this study highlights a mechanism for xenoestrogen-induced epigenetic modulation of gene expression in the uterus that correlates with developmental reprogramming of uterine leiomyoma, the elucidation of other xenoestrogen-specific mechanisms that modulate the epigenome during development will be critical to understanding risk xenoestrogens confer in increasing the susceptibility to hormone-dependent tumors.

## **CHAPTER 4: FUTURE DIRECTIONS AND CONCLUSION**

## 4.1 FUTURE DIRECTIONS

Uterine leiomyoma, like many other diseases and cancers of the reproductive tract, is a hormone-dependent disease. As discussed in chapter 1, numerous studies demonstrate that early life exposure to xenoestrogens leads to hormonal perturbations in adulthood and subsequently increases the incidence of tumor development. Studies from our laboratory (chapter 3), as well as others, have shown that neonatal exposure to xenoestrogens can disrupt normal ER-mediated signaling and induce a reprogramming effect in estrogen-responsive genes. The mechanism by which gene expression is reprogrammed is still not well understood, however, an epigenetic mechanism has been implicated in the heritable transmission of aberrant hormonal memory created by xenoestrogen exposure during development of the reproductive tract.

There is a paucity of research supporting the link between early life xenoestrogen exposure and epigenetic alterations that increase disease susceptibility. The epigenetic mechanism responsible for xenoestrogen-induced developmental reprogramming that has been focused primarily is alterations DNA methylation given that xenoestrogens, such as DES, have little effect on genetic mutations (288). A few key studies have been successful in demonstrating this link, including the study from Tang et al. (174), which showed that neonatal exposure in CD-1 mice to DES or GEN induces steroid-hormone dependent epigenetic changes that persist throughout life and increase the incidence of uterine adenocarcinoma. The chromatin-remodeling gene *Nsdp1* was found to have a low level of DNA methylation neonatally, which became further hypomethylated only after DES or GEN exposure that correlated with its overexpression in the uterus. Additionally, mice exposed neonatally to E2 or BPA increased susceptibility to neoplastic transformation of the prostate, prostatic intraepithelial neoplasia (PIN) (175). DNA hypomethylation of the gene *PDE4D4*, which accumulated DNA methylation with age in controls, was hypomethylated by estrogen or xenoestrogen exposure that prevented the gene silencing. Importantly, epigenetic changes associated with developmental reprogramming of gene expression were identified prior to the formation of PIN lesions. Likewise, we show that

reprogramming of uterine leiomyoma-specific gene expression in adult myometrium by neonatal exposure to xenoestrogens occurred in a xenoestrogen-specific manner prior to the formation of uterine tumors (Chapter 3). The reprogramming effects of both DES and GEN were the increased expression of estrogen-responsive genes, such as *Gria2*, while the opposite was true of BPA. Given the similar effects of DES and GEN on chromatin remodeling in the neonatal uterus, as opposed to BPA, leads us to next ask whether this early chromatin remodeling event induces permanent changes in DNA methylation that reprograms gene expression.

#### **4.1.1 Future Directions to Determine Xenoestrogen-Induced DNA Methylation of Developmentally Reprogrammed Genes**

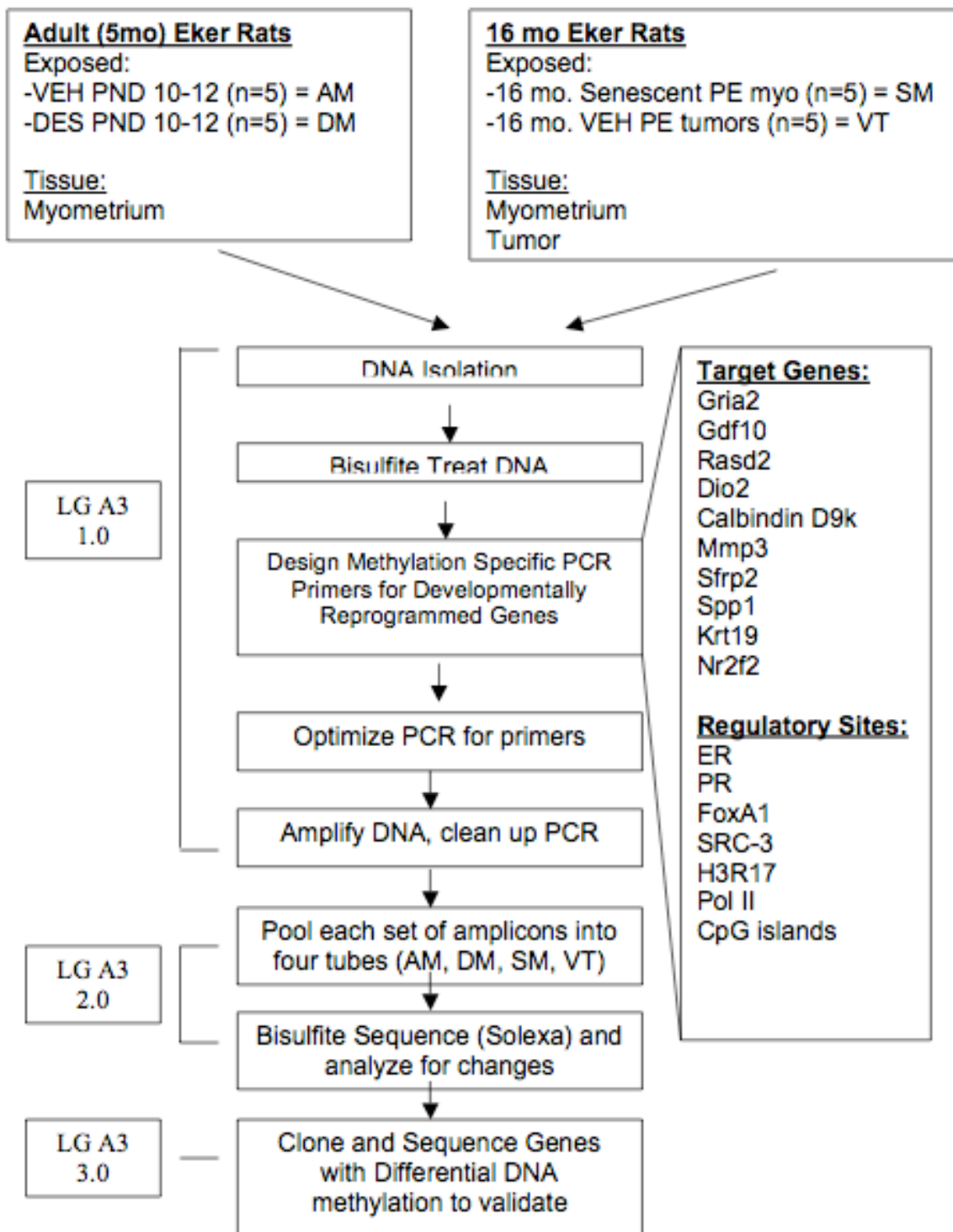
Collectively, the studies presented herein demonstrate that xenoestrogens developmentally reprogram estrogen-responsive gene expression, which is preceded by neonatal modification of a repressive epigenetic mark via xenoestrogen-specific non-genomic signaling. Thus, we hypothesize that the mechanism resulting from this early chromatin remodeling that promotes reprogramming of these genes is differential DNA methylation (Aim 3), which is initiated after exposure to xenoestrogens, established during puberty and persists into tumorigenesis. This theory would establish xenoestrogens as epigenetic “reprogrammers” of cancer risk, evidence of which has been illustrated in the studies discussed above. Therefore, the purpose of this future study is to determine if developmentally reprogrammed genes identified in our previous studies have aberrant DNA methylation profiles in regulatory regions that are controlled by xenoestrogen-induced signaling.

##### **4.1.1.1 EXPERIMENTAL DESIGN OF ULTRADEEP BISULFITE SEQUENCING IN DEVELOPMENTALLY REPROGRAMMED GENES**

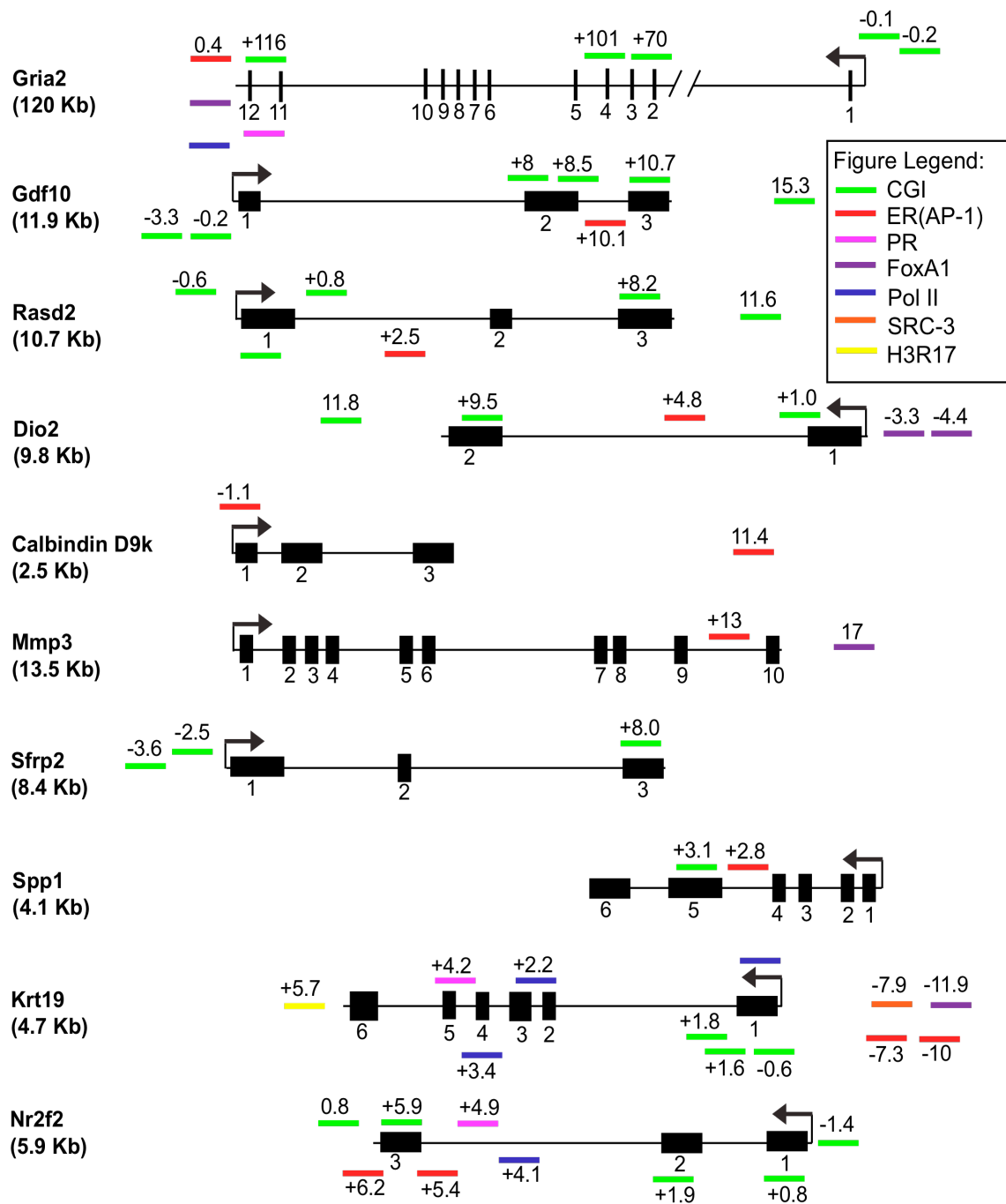
In order to address the above hypothesis, next generation bisulfite sequencing of developmentally reprogrammed genes has been conducted using primers designed to amplify bisulfite-treated DNA in myometrium and tumors. The results from this DNA methylation profiling experiment will lead to an enhanced understanding of the regions

in developmentally reprogrammed genes susceptible to xenoestrogen-induced DNA methylation alterations.

Ultradeep bisulfite sequencing of 7 regulatory regions (CpG islands (CGI), ER, PR, FoxA1, H3R17, SRC-3 and Pol II) in 10 developmentally reprogrammed genes (*Gria2*, *Gdf10*, *Calbindin D9k*, *Rasd2*, *Mmp3*, *Krt19*, *Sfrp2*, *Spp1*, *Dio2*, *Nr2f2*) was accomplished via massively parallel sequencing-by-synthesis (Solexa sequencing platform). DNA samples from 20 individual animals (AM-5 month adult VEH myometrium (n=5), DM-5 month adult DES myometrium (n=5), SM-16 month normal senescent myometrium (n=5), VT-16 month VEH tumors (n=5)) were pooled into 4 groups after amplification of each PCR product (AM, DM, SM, VT) representing the age, stage and treatment matched tissue listed. For 5-month myometrium samples, the proliferative phase of estrous was utilized for DNA methylation analysis in a manner identical to the protocol used in chapter 2 and 3 for staging estrus in VEH and DES exposed animals (AM and DM). Similarly, animals at 16 months in the PE phase of estrus (as described in chapter 2 and 3) were utilized for analysis of DNA methylation. After amplicons were generated for each regulatory region for developmentally reprogrammed genes, each amplicon was visualized by gel electrophoresis for size determination, followed by pooling and purification (**Figure 4.1**). A total of 62 primers pairs were used to interrogate the DNA methylation differences in adult and 16 month myometrium, as well as, tumors in regulatory regions of developmentally reprogrammed genes (**Figure 4.2**) as described in Appendix B “Methods for Ultradeep Bisulfite Sequencing Design and Analysis”. Importantly, these primers can also be used in the future for ultradeep bisulfite sequencing analysis of GEN and BPA exposed myometrium, however whole genome bisulfite sequencing (Bis-seq) would also be useful giving an unbiased approach.



**Figure 4.1 Experimental Design for Ultradeep Bisulfite Sequencing.** Experimental work flow for analysis of methylation in developmentally reprogrammed genes via massively parallel sequencing (Solexa) in adult and senescent myometrium or tumors.



**Figure 4.2 Primer Design for Developmentally Reprogrammed Genes.** Primers were designed to amplify bisulfite treated DNA from myometrium or tumors. Locations of primers are designated from the transcription start site (estimated or known) of each gene. Figure legend denotes the regulatory regions in which the primers were designed based off of previous ChIP-Chip data.

#### **4.1.1.2 QUANTITATIVE SEQUENCING ANALYSIS AND COMPARISONS OF DNA METHYLATION IN DEVELOPMENTALLY REPROGRAMMED GENES**

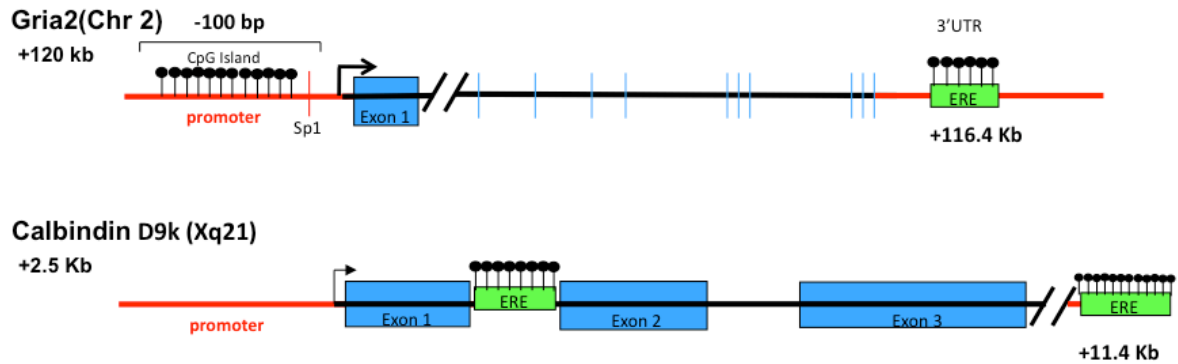
Following ultradeep bisulfite sequencing, sequences will be aligned using BSMAP (289) bisulfite mapping software. Aligned sequences will be interrogated for changes in DNA methylation between samples using the chi square statistical method.

In order to answer the question regarding normal age-related changes in DNA methylation that are independent of xenoestrogen exposure or tumor development, DNA methylation between aged normal myometrium and VEH adult myometrium (SM vs AM) should be compared. This comparison would allow us to determine which genes and/or regulatory regions are undergoing age-related changes in methylation. Subsequently we could compare the age-related methylation changes to tumor-specific changes in methylation (SM vs. VT). This comparison would aid in the understanding if genes differentially expressed in tumors acquire changes in methylation that are susceptible to changes in hormones given that these are hormone-dependent tumors. Identification of genes that are susceptible to hormonal changes in DNA methylation as opposed to those genes that undergo methylation alterations as a function of tumorigenesis or clonal expansion, would help uncover gene and regulatory sites that are most susceptible to early xenoestrogen exposure. Additionally, changes in methylation would also be compared to the gene expression changes in tumors. Necessarily, we would compare VEH and DES myometrium to determine the changes in methylation induced by neonatal exposure to DES. These DES-related changes in methylation would then be compared to the gene expression changes, which would demonstrate the relationship between a loss of methylation at the promoter of a gene and the subsequent increased gene expression. More specifically, we could also determine if changes in gene expression are correlated with a specific regulatory site beyond the canonical promoter CpG island (i.e. ER, PR, FoxA1, SRC-3, Pol II, H3R17) in DES myometrium and tumor samples. Identification of specific regulatory sites that are altered in DNA methylation may provide clues to the mechanism(s) by which xenoestrogens developmentally reprogram gene expression and alter tumor susceptibility.

#### 4.1.1.3 VALIDATION OF ULTRADEEP BISULFITE SEQUENCING BY CLONING AND SEQUENCING OF DEVELOPMENTALLY REPROGRAMMED GENES: PRELIMINARY DATA

Comparison of the DNA methylation results from bisulfite sequencing will be accomplished via traditional cloning and sequencing of gene regions identified to have differential methylation related to gene expression in adult animals (AM vs. DM). Once methylation changes are validated, DNA from DES exposed PND 12 uteri ( $n \geq 5$  VEH;  $n \geq 5$  DES), PND 21 ( $n \geq 5$  VEH;  $n \geq 5$  DES), 5 month – SM and tumors – VT should be examined for DNA methylation changes via cloning and sequencing ( $n \geq 20$  clones per sample/site) to determine the dynamics in methylation of DES-induced developmental reprogramming at critical stages including development, puberty, adulthood and during tumorigenesis. This analysis would allow us to understand the critical time points of methylation susceptibility in the myometrium to xenoestrogen exposure. As such, these same parameters could also be applied to investigation of the changes in methylation after Bis-seq in GEN and BPA exposed myometrium.

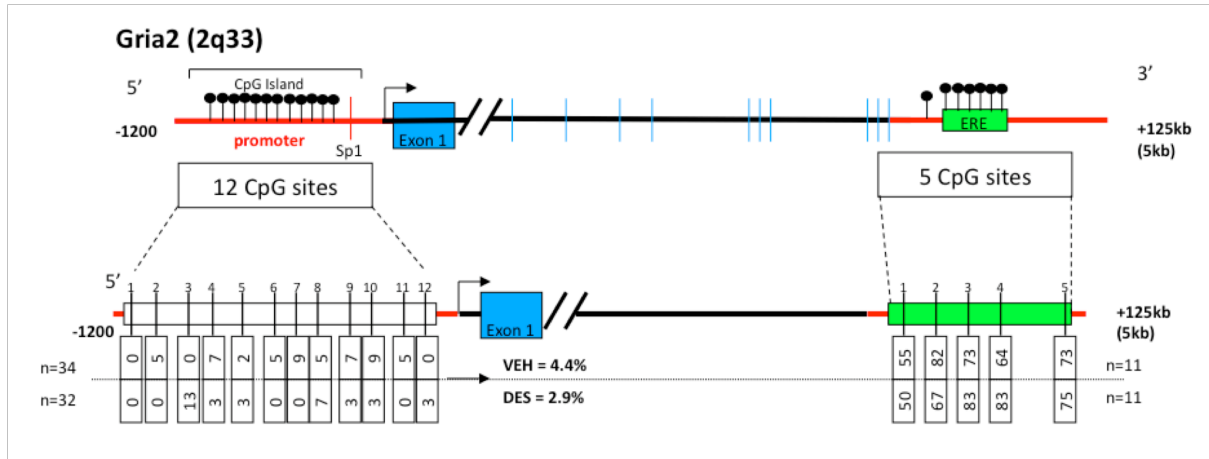
Preliminary data, which supports the mechanism of DES-induced methylation alterations, has been gathered for 2 DES-induced developmentally reprogrammed genes *Gria2* and *Calbindin D9k*. Two regions in *Gria2*, a promoter CGI and an ER binding site located 3' to the gene (**Figure 4.3**), were cloned and sequenced using the same BS-DNA samples and primers as used for ultradeep bisulfite sequencing. In a similar manner, an ER binding site located between exons 1 and 2 was also cloned and sequenced for validation (**Figure 4.3**).



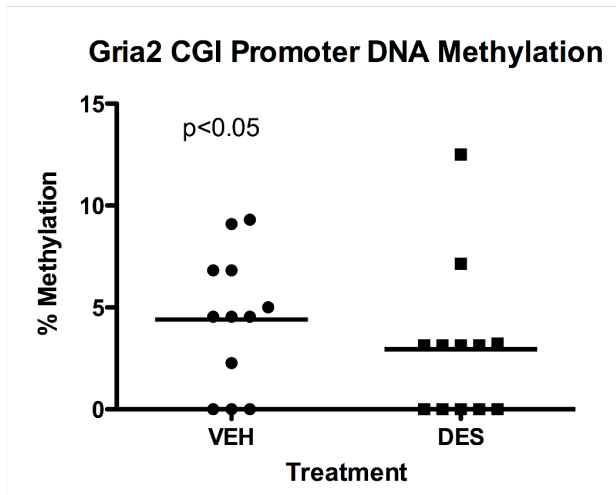
**Figure 4.3 Annotation of promoter CpG islands and EREs in *Gria2* and *Calbindin D9k*.** The CpG island in the promoter of *Gria2* contains 12 CpG sites the ERE 6 CpG sites around which bisulfite specific primers were designed. For *Calbindin D9k*, 2 EREs with 8 and 12 CpG sites, respectively, were used for bisulfite-specific primer design.

Quantitative examination of DNA methylation in the promoter CGI of *Gria2* showed a significant hypomethylation in DES vs. VEH animals among 12 CpG sites (**Figure 4.4**) that corresponded to the increased gene expression seen in adult myometrium after neonatal DES exposure as demonstrated in chapter 2. However, analysis of the ERE (5 CpG sites) 3' of *Gria2* did not show any significant difference in overall or site specific methylation between DES and VEH samples (**Figure 4.4**). Similar to *Gria2* expression, the expression of *Calbindin D9k* was increased in its expression after DES exposure as compared to VEH (Chapter 2, Figure 2.3). While overall DNA methylation analysis of the ERE (8 CpG sites) in *Calbindin D9k* was not significantly different between DES and VEH myometrium, site-specific differences were observed. Specifically, CpG site 1-3 were found to be significantly hypermethylated, while CpG site 6 was significantly hypomethylated in DES samples as compared to VEH (**Figure 4.5**). Though it remains unclear as to the impact of ERE CpG methylation on gene expression, a plethora of data show a significant correlation between promoter hypomethylation and increased gene expression. For this reason, it will be important to examine the changes in DNA methylation at they relate to gene expression changes seen in developmentally reprogrammed genes.

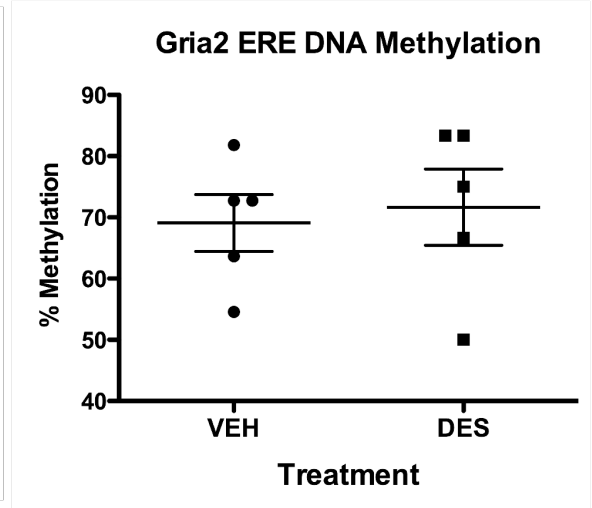
**A**



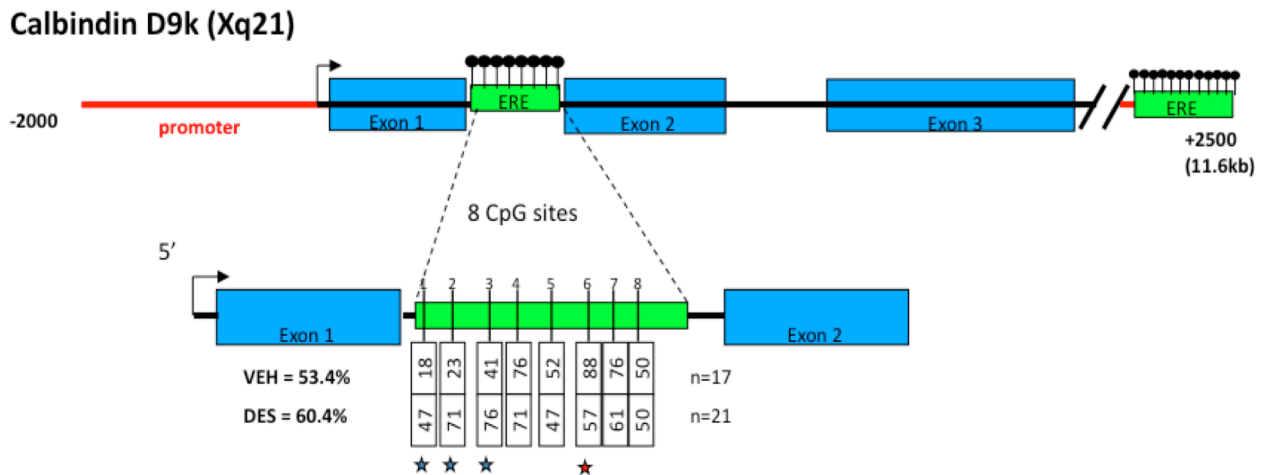
**B**



**C**

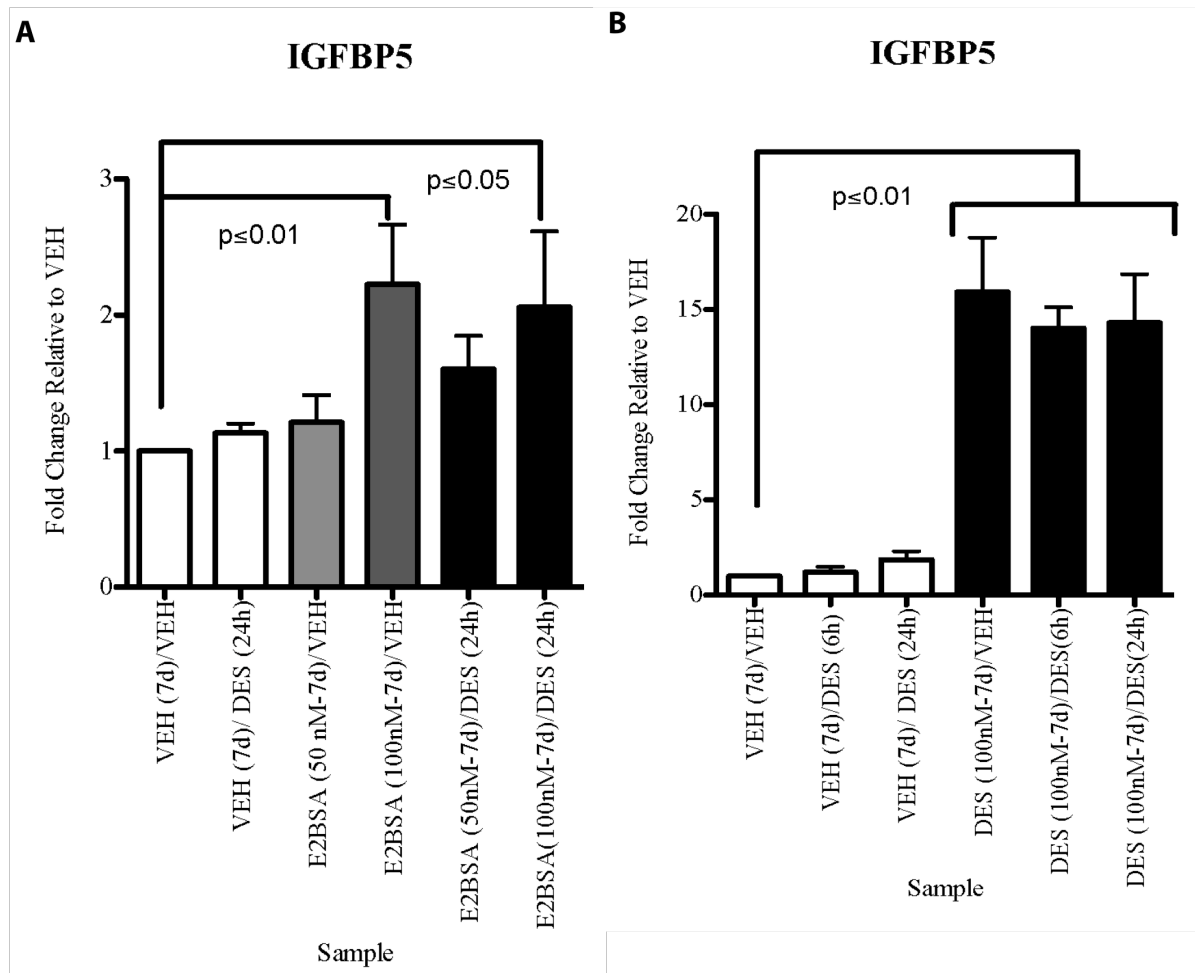


**Figure 4.4. DNA Methylation Analysis of Gria2 in DES Exposed Adult Myometrium.** Myometrium from 5 month old Eker rats exposed to DES or VEH on PND 10-12 were used for bisulfite treatment of DNA. A) Amplicons from the CpG island of Gria2 were cloned and sequenced for the presence of DNA methylation alterations. B) DNA methylation in DES myometrium was significantly different as compared to VEH myometrium, Students t-test  $p<0.05$ . C) No significant difference was observed at CpGs sites at the ERE in Gria2.

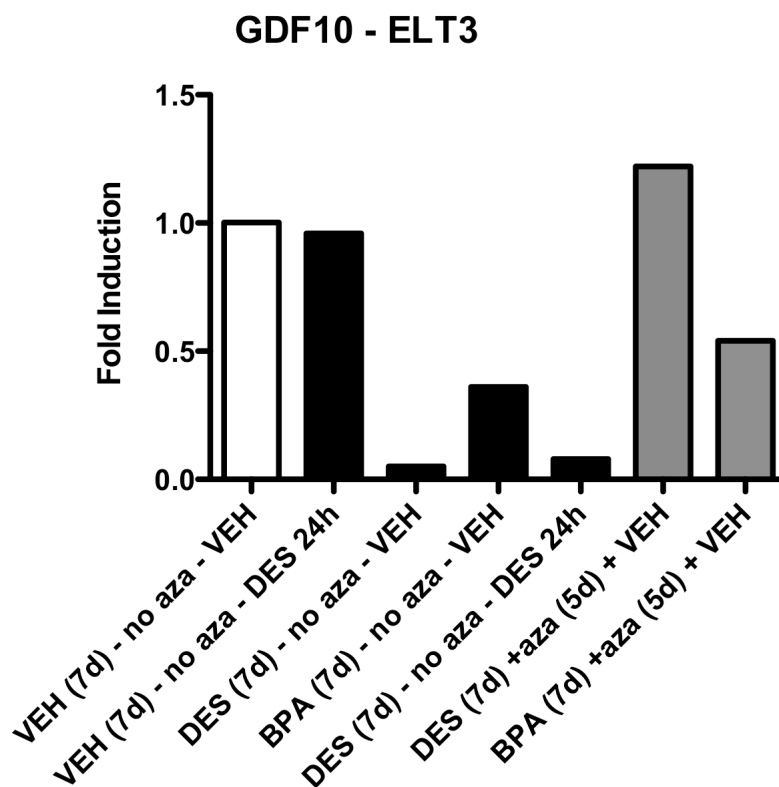


**Figure 4.5. DNA Methylation Analysis of Calbindin D9k.** DNA isolated from adult Eker rats exposed to DES or VEH on PND 10-12 were treated with sodium bisulfite. DNA containing 8 CpG sites in the ERE regions was amplified, cloned and sequenced. CpG sites 1-3 demonstrated significant hypermethylation in DES vs. VEH myometrium, blue stars,  $p < 0.05$  Student's t-test. CpG site 6 demonstrated hypomethylation, red star,  $p < 0.05$ .

The question regarding the relationship between changes in DNA methylation and developmental reprogramming of gene expression could be addressed using qPCR in combination with deletion constructs. The experiment would involve 1) identifying critical regions that undergo changes in DNA methylation that correlate with gene expression changes (induced or repressed) from Bis-seq and 2) using the myometrial tumor cell line, ELT3, cells exposed to xenoestrogens (DES, GEN or BPA) or VEH for 7days to reprogram gene expression as demonstrated by Bredfeldt *et al.* (271) (**Figure 4.6**). These cells would then be treated with DMSO or 5 deoxy-azacytidine (5 aza) for 3 days (25  $\mu$ M), which is incorporated into DNA and prevents DNA methylation during replication by creating a covalent link between 5 aza-CpGs and Dnmt1 resulting in loss of methylation and, in general, activation of silenced genes (145). RNA would then be harvested from treated cells and qPCR would be conducted to determine if treatment with 5 aza could reverse the reprogramming of gene expression induced by “priming” with xenoestrogens. Preliminary data for the developmentally reprogrammed gene *Gdf10* has been conducted and results demonstrate *in vitro* that *Gdf10* is not induced in expression initially by a brief 24 hr DES exposure in comparison to VEH (**Figure 4.7**). However, prolonged exposure to DES, and to a lesser extent BPA, represses gene expression. Interestingly, after priming with DES treatment, 5 aza reversed the DES-induced repression of *Gdf10* and partially relieved the BPA-induced repression. To determine if these gene expression changes are related to changes in DNA methylation, primers designed for Bis-seq could be used to interrogate the DNA methylation in regulatory regions of genes reprogrammed by DES and reversed by 5 aza. These data indicate potentially that the expression of developmentally reprogrammed genes can be “reset” after xenoestrogen exposure by inducing changes in DNA methylation at regions critical to regulating gene expression.



**Figure 4.6 Estrogen priming of ELT3 cells reprograms gene expression.** A) ELT3 cells were treated for 7 days with VEH (PBS) or E2 conjugated to BPA. 48 hrs after removal of treated or untreated media, or recovery, cells were treated with DES for 24hrs (50nM) and RNA was isolated for qPCR. B) ELT3 cells were treated for 7 days with VEH or DES (100 nM), allowed to recover and then treated with DES for 6 or 24 hrs. RNA was isolated and qPCR was performed. Student t-test was performed to determine significance, which was set at  $p \leq 0.05$ .



**Figure 4.7 Reprogramming of *Gdf10* gene expression by priming with xenoestrogens is reversed by 5 deoxy-aza-cytidine treatment in ELT3 cells.** ELT3 cells were treated with VEH, DES or BPA for 7 days followed by VEH (DMSO) or 5 deoxy-aza-cytidine for 5 days. After treatment with VEH or DES for 24 hrs, RNA was isolated and qPCR for *Gdf10* was conducted. Fold inductions was calculated using the reference gene *18s* and calibrated to VEH – no aza – VEH.

To determine if the regions identified in the above DNA methylation studies and gene expression studies with 5 aza are indeed regulated in their expression by DNA methylation at regulatory regions (previously identified above), deletion constructs could be generated that are representative of regions in the gene under investigation. For example, if a region within *Gdf10* was found to be differentially methylated and responsive to 5 aza treatment, as demonstrated, then deletion constructs could be generated for these regions and subcloned upstream of a luciferase reporter construct (as described in (290)). This luciferase deletion construct would then be grown up in media containing M.SssI (CpG methyltransferase) or no enzyme plus SAM (mock methylation) creating an unmethylated or methylated version of the deletion construct to be used in transient transfections of ELT3 cells (as described in (291, 292)). Following transfection, priming with DES or VEH would occur and cells would be subjected to luciferase analysis to determine if prior methylation status and/or treatment with DES (or VEH) changed expression of this critical region of *Gdf10*. This experiment would, therefore, allow us to answer the question whether DNA methylation changes induced by xenoestrogens directly regulate the expression of developmentally reprogrammed genes.

#### **4.1.2 Future Directions to Determine the Mechanism(s) of Non-Genomic Signaling and Chromatin Remodeling by Xenoestrogens in Developmentally Reprogrammed Genes**

To expand on Aim 3 and investigate mechanisms governing alterations in chromatin remodeling and DNA methylation, it would be prudent to determine the non-genomic effects of xenoestrogens *in vivo* in the absence of ERE-dependent genomic signaling, given that xenoestrogens have unique effects on non-genomic signaling (Chapter III). These studies would also be the logical next step to establish biological relevance of *in vitro* non-genomic ER signaling that results in chromatin modifications via AKT-induced phosphorylation of EZH2. Subsequently, to further delineate the

plasticity and dynamics of chromatin remodeling effects of xenoestrogen-induced non-genomic signaling, experiments should be conducted to monitor changes in binding of EZH2 to chromatin and in levels of H3K27me3 in regulatory regions of developmentally reprogrammed genes. Alternatively, other non-genomic signaling pathways that are activated by xenoestrogens, which alter chromatin conformation, DNA methylation and gene transcription should also be examined, such as the PKA-induced phosphorylation of methyl binding protein 4.

#### **4.1.2.1. IN VITRO AND IN VIVO EXAMINATION OF XENOESTROGEN-SPECIFIC NON-GENOMIC SIGNALING AND CHROMATIN REMODELING**

Collectively, the results from our studies presented herein show that 1) non-genomic signaling activates AKT and induces chromatin remodeling via phosphorylation of EZH2 in an ER-dependent mechanism, and 2) activation of AKT and phosphorylation of EZH2 *in vivo* occurs in a xenoestrogen-specific manner. Because of the plasticity of not only the developing uterus and HPG axis, but also the chromatin environment, the elucidation of the effects of neonatal xenoestrogen exposure on non-genomic ER-dependent signaling would more precisely define the effects on the epigenome. Two important studies, (293) and (294), demonstrate the importance of non-genomic ER signaling using animal models of DNA binding domain (DBD) deficient ER $\alpha$  mice (ER<sup>AA</sup>) in which the zinc finger domain of ER $\alpha$  has been mutated so that mice are either deficient in ERE-dependent genomic signaling (ER<sup>-</sup>/<sup>AA</sup>) or have reduced genomic signaling abilities (ER<sup>+/AA</sup>). Interestingly, ER<sup>+/AA</sup> mice have reproductive tract morphological alterations that are reminiscent of the effects observed in neonatal DES exposed animals, including infertility, lack of ovarian corpora lutea and endometrial hyperplasia. Establishment of the HPG axis is also dependent on ER-dependent non-genomic signaling as demonstrated in ER<sup>-/AA</sup> mice, which have no wild-type (WT) copy ER $\alpha$  and one mutate (DBD) copy of ER $\alpha$ . This study shows that non-genomic activation of p21-activated kinase 1 (PKA1) in the brain is an important regulator of E2-induced negative feedback given that both WT (ER<sup>+/+</sup>) and ER<sup>-/AA</sup> could activate PKA1 and suppress LH after E2 treatment in contrast to ER<sup>-</sup>

<sup>1/-</sup> mice. These studies illustrate that non-genomic ER-dependent signaling events govern the proper development of the reproductive tract and HPG axis that are independent of ERE-dependent genomic signaling. Furthermore, they suggest two possible hypotheses of xenoestrogen induction of ER-dependent non-genomic signaling. One, potentially the balance of genomic and non-genomic signaling is highly susceptible to perturbation by xenoestrogen exposure during development. Two, it is possible that the non-genomic signaling pathway is more active during reproductive tract development as compared to genomic signaling and as such xenoestrogen-specific effects are more pronounced.

In order to address the remaining questions of the effects regarding xenoestrogen-induced non-genomic signaling in the absence of ERE-dependent signaling, the ER<sup>-/-AA</sup> mouse model could be used. We have shown that membrane-bound (E2-BSA) ER signaling *in vitro* activates non-genomic ER signaling and results in chromatin modification (i.e. levels of H3K27me3). However, the potential ERE-independent mechanisms have not been explored *in vivo*. ER $\alpha$ <sup>-/-AA</sup> and ER $\alpha$ <sup>+/-</sup> mice would be exposed neonatally to DES (1mg/kg), GEN (50 mg/kg) or BPA (50 mg/kg or 50 ng/kg) on PND 1-5 (a window of uterine development shown to be susceptible to xenoestrogen exposure in CD-1 mice) or VEH. In addition to the examination of the developmental reprogramming of gene expression in the adult myometrium (via microarray and/or qPCR), the non-genomic ER signaling effects could then be examined via western blotting of AKT, S6, EZH2 and H3K27me3 along with immunoprecipitation of pEZH2 in uteri of neonatal animals. It would also be important to determine the morphological alterations (e.g. ovaries, uterus and vagina) induced in these animals by xenoestrogen exposure, which would distinguish the non-genomic ER $\alpha$ -dependent developmental reprogramming effects from those mediated by ERE-dependent genomic signaling.

To further explore the xenoestrogen-induced non-genomic signaling effects on chromatin remodeling, experiments should be designed to address the following hypothesis. The modulation of EZH2 activity and transient decrease of H3K27me3 levels is a mechanism by which permanent changes in DNA methylation at critical regulatory regions is induced resulting in the developmental reprogramming of uterine gene expression. It would be necessary determine first, in regions of developmentally

reprogrammed genes found to have reduced levels of DNA methylation, if H3K27me3 levels were also reduced. This could be accomplished using chromatin immunoprecipitation (ChIP) with the antibody against H3K27me3 (as described in (295)) in protein lysates of neonatally xenoestrogen exposed uteri. Briefly, minced uteri would be cross-linked with 1% formaldehyde and quenched with 0.125 M glycine to fix chromatin and protein. Cells would be lysed with acid washed glass beads and sonicated briefly to shear fixed chromatin into small pieces (400-800 bp) prior to immunoprecipitation with  $\alpha$ -H3K27me3. Cross-linked immunoprecipitated H3K27me3 chromatin would be reversed to yield DNA free of histones for purification and PCR. PCR of purified DNA with primers designed within regions of developmentally reprogrammed genes previously identified as having reduced DNA methylation after xenoestrogen exposure would be performed. The data from this experiment would show the amount of H3K27me3 present in these critical gene regions after xenoestrogen exposure.

It would also be important to determine if these changes in H3K27me3 are transient or permanent in critical regulatory regions of developmentally reprogrammed genes as a result of xenoestrogen exposure. As discussed in chapter 2, the *Tat* gene undergoes chromatin remodeling after glucocorticoid exposure followed by (>2-3 d) loss of DNA methylation at a regulatory site and up-regulation of *Tat* expression. However, after chromatin reverts back to its initial state of conformation, the changes in DNA methylation remain. Initially, it would be necessary to define when estrogen-responsive genes become reprogrammed. Using Eker rats exposed on PND 10-12 to DES (or VEH) and re-exposed to DES (or VEH) on PND 21, myometrium would be analyzed for changes in gene expression of developmentally reprogrammed genes (e.g. *PR*, *Gria2*). These data would delineate the critical time of gene reprogramming, that is, if endogenous hormones “re-set” the expression of estrogen-responsive genes or if it is established soon after genomic signaling is down-regulated following xenoestrogen exposure. From these data, experiments could be designed using ChIP to examine the dynamics of H3K27me3 in critical regions of developmentally reprogrammed genes. This would necessitate investigating time points that correspond with rapid changes in chromatin remodeling and transcription, 30 min to 24hrs, as compared with more permanent changes that may occur as a result of

reprogramming after several days. Together, these data would link changes in non-genomic signaling with changes in H3K27me3 at critical regions in developmentally reprogrammed genes that become hypomethylated as a result of xeneostrogen exposure.

## **4.2 CONCLUSIONS AND IMPLICATIONS**

Developing organisms have evolved several conserved mechanisms among species in order to adapt to changing environments or stress. However, these mechanisms that allow environmental adaptation to stressors can be overcome leading to various disorders and potential teratogenicity. The ability to buffer these environmental stressors suppresses morphological variation in favor of canalization (296). These emergency and adaptive mechanisms consist of essentially three main facets (297): placental toxicant defenses, metabolic adaptations and hormonal buffering. Placental toxicant defenses, such as efflux transporters (298) and antioxidants, prevent or buffer the passage of environmental toxins from passing the placental barrier. Thalidomide, however, the developmental teratogen, can bypass this buffering mechanism (299, 300). As described previously, metabolic adaptations or maladaptations that exceed the metabolic buffering system, such as malnutrition during pregnancy can result in offspring with a “thrifty phenotype” or that are metabolically hypersensitive giving rise to Type II diabetes and cardiovascular disease. Hormonal perturbations during development, including xenoestrogen exposure, can bypass hormone binding proteins, such as alpha-fetoprotein that exist to bind and inactivate endogenous estrogens that allow for normal reproductive tract formation. When environmental stressors surpasses these buffering systems, developmental reprogramming can occur, which leads to morphological alterations and development of adult diseases.

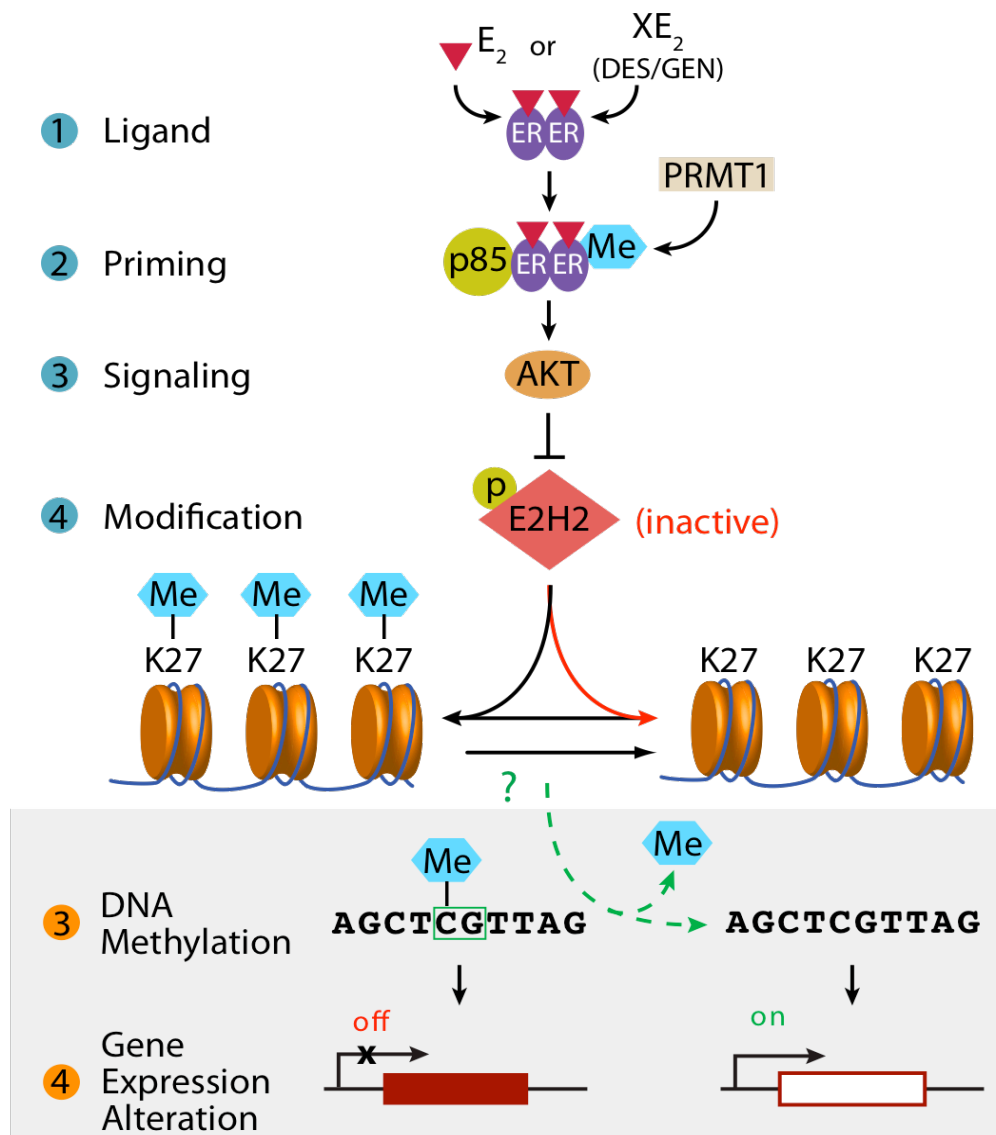
As addressed throughout this work, neonatal xenoestrogen exposure induces developmental reprogramming of the reproductive tract and increases cancer risk, as demonstrated by our laboratory and others. Developmental exposure to EDCs, such as xenoestrogens, disrupts normal reproductive tract development and increases the risk for cancer in both human and animal models. While the developmental reprogramming effects of DES have been investigated since the 1970s, the ability of developmental

exposure to xenoestrogens GEN and BPA have been hotly debated, with the later coming under heavy scrutiny. However, not all xenoestrogens have the same effect though all are classified as having “estrogenic” effects. Understanding how each xenoestrogen induces developmental reprogramming is key to determining how cancer risk is affected and if later generations are at risk. For example, the structures of DES, GEN and BPA have similarities and differences, which may relate to their affinity to ER as well as to their ability to bind to specific isoforms of ER or induce unique dimerization complexes. The xenoestrogen-specific binding and dimerization conformation differences may also constitute the variations observed in non-genomic signaling in the neonatal uterus. In contrast, however, all three xenoestrogens were effective in activating the PI3K/AKT pathway in both MCF-7 cells (93). These data suggest that developmentally-specific genes or miRNAs may be expressed that change how signals from xenoestrogens are transmitted, further illustrating the importance of understanding the differences in the mechanism among the xenoestrogens during development. Unfortunately, very little is still known regarding the mechanisms of developmental reprogramming by xenoestrogens or how they confer an increased incidence of uterine tumors. As such, the main focus of this work has been on identifying gene targets for developmental reprogramming by xenoestrogens that are associated with uterine leiomyomas, as well as, the mechanisms utilized by xenoestrogens to induce developmental reprogramming at the molecular and morphological levels.

In Specific Aim 1 we endeavored to identify estrogen-responsive genes that were being reprogrammed as the result of xenoestrogen exposure, which may confer an increased susceptibility to uterine leiomyoma in the genetically susceptible Eker rat. From this gene profiling study we found that several genes associated with uterine leiomyoma development that are developmentally reprogrammed in their response to estrogen after neonatal DES exposure. Importantly, 5/6 of these genes that were overexpressed in tumors were all induced in their expression prior to the onset of tumorigenesis indicating that DES induces an aberrant hormonal environment that promotes tumorigenesis above that which would occur spontaneously. Using these gene candidates identified in Aim 1, we next asked in Aim 2 whether other environmentally relevant xenoestrogens, GEN and BPA, also modulated tumor incidence via developmental reprogramming of estrogen-responsive genes.

Surprisingly, we found that GEN and BPA had opposite effects on reprogramming of gene expression. While GEN exposure, like DES, developmentally reprogrammed common genes, the effect was opposite of BPA. Among the genes reprogrammed by BPA, the general effect was repression, in contrast to DES and GEN. Interestingly, these opposing effects of GEN and BPA were correlated with their effects on tumorigenesis, such that GEN (and DES) significantly increased tumor incidence above the spontaneous rate, while BPA did not modulate tumorigenesis, which was consistent with the developmental reprogramming effects. These data demonstrated that there are xenoestrogen-specific effects on developmental reprogramming of gene expression that correlates with the ability to modulate tumor incidence. The differences observed in this study were intriguing and lead us to investigate the mechanisms governing the differences we observed between these xenoestrogens.

We were able to determine a novel mechanism of how xenoestrogen exposure could result in contrasting reprogramming effects. In chapter 3, we demonstrated that the transmission of estrogen-induced signaling into histone modifications occurs through interaction of xenoestrogen/E2-bound ER with the ER/PI3K/AKT pathway. Furthermore, this pathway of xenoestrogen-induced histone modification occurred *in vivo* and was ER-dependent. We also demonstrated *in vitro* the link between DES exposure, or “priming”, and the reprogramming of estrogen-responsive genes that were known to be susceptible to developmental reprogramming. Importantly, EZH2 was not phosphorylated after BPA exposure in comparison to DES and GEN. These results indicate that, while xenoestrogens may have estrogenic properties, during development of the reproductive tract they have very different effects on non-genomic signaling that ultimately result in chromatin remodeling. Using our data, together with data from another laboratory showing the importance of priming of ER $\alpha$  by the protein arginine methyltransferase, PRMT1(301), we developed a new model of non-genomic ER-dependent signaling that changes the activity of EZH2 and, thus, the levels of H3K27me3 in xenoestrogen-specific manner. With the future directions of this project it will possible to determine if these chromatin modification also change DNA methylation and gene expression (**Figure 4.8**).



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**Figure 4.8 Proposed mechanism of xenoestrogen-induced developmental reprogramming of gene expression.** Based on work from our laboratory and others it is now known that  $E_2$  and xenoestrogens (DES/GEN) 1) bind ER, which is 2) primed by PRMT1 and 3) leads to activation of non-genomic signaling. 4) Phosphorylation of E2H2 by AKT reduces its activity resulting in loss of methylation on H3K27. Still unknown, however, is whether this loss of histone methylation contributes to a loss of DNA methylation in estrogen-responsive genes and subsequent alterations in gene expression.

Understanding how reprogramming of gene expression occurs is a fundamental question related to the mechanism of xenoestrogen-induced developmental reprogramming and the disparate effects seen among the xenoestrogens. To address this question we hypothesized in Aim 3 that neonatal xenoestrogen exposure induced alterations in DNA methylation in developmentally reprogrammed genes. EDCs, including xenoestrogens, have transgenerational effects in both human and animals studies, which is indicative of an epigenetic mechanism of reprogramming. Developmental changes in DNA methylation induced by aberrant hormone or xenoestrogen exposure changes the response to subsequent hormone exposure. Pre- and postnatal DES exposure leads to an increased incidence of uterine tumors in F2 generations (9), which has also been demonstrated for the EDC vinclozolin (180). Importantly, developmental reprogramming of spermatogenesis in later generations was found to be correlated with changes in DNA methylation (182). DES has also been demonstrated to result in permanent alterations in DNA methylation and gene expression (i.e. *c-fos* and *Nsbp1*). For this reason, it is also possible that xenoestrogens which induce changes in DNA methylation can also transmit the increased risk to subsequent generations. In addition, the heritable nature of DNA methylation, as well as, histone modifications, though less well understood, makes it an obvious candidate for the transmission of information gathered from hormonal signals during development into permanent physiological responses in adults. Furthermore, if this aberrant exposure to xenoestrogens occurs during gametogenesis, then alterations would be passed down to subsequent generations. Therefore, it is important to determine the molecular mechanisms of the xenoestrogens that lead to DNA and histone modifications. The experiments to examine this mechanism, which are outlined above, have yielded preliminary results that show a significant hypomethylation in the promoter of the developmentally reprogrammed gene, *Gria2*, in the myometrium of DES exposed animals. These alterations, which are likely induced by transient histone modifications in chromatin may leave permanent changes in DNA methylation in key regions of gene regulation like *Gria2*.

Given the plasticity of the chromatin environment, stressors in the form of aberrant hormone exposure may “re-set” the normal gene expression response to endogenous hormone via changes in histones modifications that alter DNA methylation and gene expression. This new gene expression pattern could create an

environment that is hyper-responsive to hormones and, therefore, more susceptible to hormone-dependent tumors. However, we demonstrate that this reprogramming event is xenoestrogen-specific such that DES and GEN are capable of engaging the non-genomic chromatin-remodeling pathway via PI3K/AKT in the developing uterus, while BPA does not. However, it would be prudent to further investigate the pathways of BPA-induced developmental reprogramming given that gene expression was altered in the adult myometrium. Overall, we have defined a mechanism for xenoestrogen-induced developmental reprogramming via histone modification and are working towards reconciling this pathway with changes in DNA methylation that may give rise to permanent changes in gene expression.

The implications of these findings are multi-faceted. First, the unique effects each xenoestrogen has on signaling, chromatin modification, gene expression and tumor development create a new understanding of the epigenetic mechanisms that are engaged by xenoestrogens. Interestingly, two *in vitro* studies from the Huang laboratory demonstrate that exposing mammospheres to E2 or DES changed the DNA methylation and histone modification patterns, which correlated with epigenetic pattern and gene expression in tissue from breast cancer patients (211, 302). Specifically, exposure of mammospheres to DES induced aberrant DNA methylation of miRNAs, but also altered histone marks H3K27 and H3K9. These studies link epigenetic changes induced after exposure of progenitor cells to E2 or DES with carcinogenesis. Additionally, recent evidence of the predictive value of epigenetic signatures of hormone-dependent tumors, specifically ovarian cancer, was obtained from a comparison between normal endometrial tissue and that from patients with ovarian cancer (303). It was found that in target genes of the polycomb group, *HOXA9*, *HOXA10* and *HOXA11*, increased methylation in normal endometrium was associated with a significant increased risk (12.3 – 14.8 fold) of ovarian cancer. Furthermore, *HOXA10* was also found to be hypermethylated in the uterus after *in utero* exposure to DES, which correlated with its persistent aberrant expression (172). Since we know that hormone-dependent cancers display a unique epigenetic profile (303) and that xenoestrogens induce unique epigenetic signatures in the developing reproductive tract, being able to compare these two profiles could help to predict the risk of developing hormone-dependent tumors, as well as, elucidating an enhanced understanding of the mechanism of etiology. In addition, using the data from this work,

showing the xenoestrogen-specific activation of the PI3K/AKT pathway and repression of the activity of EZH2, may be a useful tool in evaluating the potentially harmful effects of exposure to other xenoestrogens and the risk of developing hormone-dependent tumors, such as uterine leiomyoma.

Second, defining the etiology of uterine leiomyoma and increased risk of uterine leiomyoma from xenoestrogen exposure is also relevant to its prevention, early identification and/or treatment. In Chapter 2 and 3 we identified genes associated with uterine leiomyomas that are developmentally reprogrammed by neonatal exposure to xenoestrogens. Critically, exposure to DES and GEN was also correlated with increased incidence of uterine leiomyoma, unlike BPA. In light of the recent evidence of increased risk of this disease that is associated with consumption of soy formula, these results imply that, like DES, exposure to high concentrations of genistein-containing products, such as soy formula, during development should be re-evaluated as a suitable alternative for breast-feeding as mechanism of prevention. In addition, a recent transcriptional profiling study was performed using myometrium and tumors from our Eker rat model, which was compared to normal myometrium and uterine leiomyomas from patients (248). This study found several genes that were differentially expressed in our analysis as differentially expressed in both Eker rat and human uterine leiomyomas, including *Gria2*, which became hyper-responsive after DES and GEN exposure but repressed further by exposure to BPA. While the importance of *Gria2* in uterine leiomyomagenesis is poorly understood, it stands out as biomarker of this disease as shown in Table 1.3. In this study, however, they also identified a significant up-regulation in genes of the mTOR pathway in both rat and human tumors. Using the mTOR inhibitor, rapamycin (WAY-129327), they found that treatment (2 weeks or 4 months) in adult animals significantly reduced tumor incidence, volume and multiplicity. Treatment with this rapamycin analogue also ablated the expression of pS6 kinase and pS6 in ELT3 cells and pS6 in tumors from Eker rats. Given that DES and GEN activated this pathway aberrantly in the neonatal uterus, which induces chromatin modifications in H3K27me3, it is possible that activation of pS6 and reduction in H3K27me3 could be used as biomarkers of xenoestrogens that increase the risk of hormone-dependent tumors as a result of developmental reprogramming, such as uterine leiomyoma. Because of the high percentage of woman who succumb to this disease and the lack of clinically

convenient screening tools, prevention by reducing consumption of or exposure to harmful xenoestrogens during development may be a useful adjuvant in addition to clinical treatment with compounds like WAY-129327. The important and novel mechanisms of xenoestrogens demonstrated in this work have illuminated key pathways that lead to developmental reprogramming, which demonstrates the crucial need to further explore the role aberrant estrogen exposure during development plays in disease susceptibility.

APPENDIX A: IDENTIFICATION OF UTERINE LEIOMYOMA GENES DEVELOPMENTALLY  
REPROGRAMMED BY NEONATAL EXPOSURE TO DIETHYLSTILBESTROL IN REPRODUCTIVE  
SCIENCES, 2008

# Identification of Uterine Leiomyoma Genes Developmentally Reprogrammed by Neonatal Exposure to Diethylstilbestrol

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*Environmental exposures during development can alter susceptibility later in life to adult diseases including uterine leiomyoma, a phenomenon termed developmental reprogramming. The goal of this study was to identify genes developmentally reprogrammed by diethylstilbestrol (DES) and aberrantly expressed in leiomyomas. Transcriptional profiling identified 171 genes differentially expressed in leiomyomas relative to normal myometrium, of which 6/18 genes with putative estrogen responsive elements and confirmed to be estrogen-responsive in neonatal uteri were reprogrammed by neonatal DES exposure. Calbindin D9k and Dio2, normally induced by estrogen, exhibited elevated expression in DES-exposed animals during both phases of the estrus cycle. Gdf10, Car8, Gria2, and Mmp3, genes normally repressed by estrogen, exhibited elevated expression in DES-exposed animals during the proliferative phase, when estrogen is highest. These data demonstrate that neonatal DES exposure causes reprogramming of estrogen-responsive genes expressed in uterine leiomyomas, leading to over-expression of these genes in the myometrium of exposed animals prior to the onset of tumorigenesis.*

**KEY WORDS:** Uterine leiomyoma, developmental reprogramming, diethylstilbestrol, gene expression.

## BACKGROUND

The plasticity of program-directing patterns of gene expression in the developing fetus allows for adaptation of developing tissues to environmental stimuli to optimize survival of the fetus in the future environment. However, when the stimuli are adverse, this plasticity can also lead to “phenotypic modulation” or a “lack of canalization,” putting in place an unfavorable physiological program that can contribute to many adult-onset diseases.<sup>1,2</sup> The existence

of this phenomenon, termed developmental reprogramming, is supported by a significant amount of evidence demonstrating that *in utero* and perinatal exposure to a suboptimal environment such as malnutrition, hypoxia, chemicals, or ionizing radiation, at critical times during development predisposes the fetus to chronic adult diseases.<sup>3,4</sup> Numerous studies have also demonstrated that perinatal exposure to xenoestrogens, found ubiquitously in the environment, can developmentally reprogram the reproductive tract, causing alterations in morphology, hormonal milieu, and gene expression, and giving rise to adult diseases such as infertility and cancer.<sup>5-9</sup>

The association between developmental reprogramming and perinatal xenoestrogen exposure was first discovered in 1971<sup>10</sup> as a result of human fetuses being exposed to diethylstilbestrol (DES) *in utero*. Diethylstilbestrol, a pharmaceutical estrogen given to women with high-risk pregnancies from the 1940s to the 1970s to prevent miscarriage, was responsible for the induction of vaginal clear cell adenocarcinoma, an otherwise rare

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form of cancer, in the daughters of treated women.<sup>11,12</sup> These “DES daughters” also had reproductive tract malformations including vaginal adenosis, hypoplasia of the vagina and cervix, T-shaped uterus, and tubular irregularities,<sup>13</sup> accompanied by infertility.<sup>14</sup> In addition, women exposed to DES *in utero* had an increased risk for breast cancer, preeclampsia, and in some cohort studies, uterine leiomyoma,<sup>9,15–17</sup> further supporting the association between *in utero* DES exposure and adult disease.

Previous studies from our laboratory and others have demonstrated an association between early life xenoestrogen exposures, developmental reprogramming at the cellular and molecular level, and increased risk for adult disease, including uterine leiomyoma. Uterine leiomyoma are hormone-responsive tumors that arise from the smooth-muscle myometrial layer of the uterus, and are the most common tumor in women.<sup>18</sup> Understanding the potential for environmental exposures to contribute to the development of this disease is important not only to better understand disease etiology, but also to identify risk factors that may contribute to the extraordinarily high incidence of these tumors. However, little is known about genes in the myometrium that participate in the development of leiomyoma or which genes in this tissue may be targets for xenoestrogen-induced developmental reprogramming.

Using female Eker rats, which develop spontaneous hormone-responsive uterine leiomyomas due to a germ-line defect in the tuberous sclerosis complex 2 (*Tsc-2*) tumor suppressor gene, we found that neonatal xenoestrogen (DES) exposure during a critical period of uterine development (ie, postnatal days 3–12) increased the incidence of leiomyoma from ~65%, to between 95% and 100%.<sup>19,20</sup> Furthermore, in rats exposed to DES neonatally, *Calbindin D9k* and progesterone receptor (*Pgr*) expression became hyper-responsive to estrogen in adult females, indicating that neonatal DES exposure had reprogrammed gene expression in the myometrium of adult animals.

Here we report a microarray-based analysis of age-matched normal myometrium and uterine leiomyomas from Eker rats where we identified 171 genes differentially expressed in tumor versus normal tissue. Because leiomyomas are estrogen-dependent tumors, we focused on differentially expressed genes that contained a putative estrogen response element (ERE), and examined a subset of these as candidates for developmental reprogramming. Out of 19 of these candidate genes, 18 were validated as being estrogen responsive and differentially expressed in tumors by real-time polymerase chain reaction (PCR).

In all, 6/18 of these genes (*Gdf10*, *Car8*, *Calbindin D9k*, *Dio2*, *Gria2*, and *Mmp3*) were subsequently identified as being reprogrammed in the myometrium by neonatal exposure to the xenoestrogen DES. Furthermore, 5/6 (*Gdf10*, *Car8*, *Dio2*, *Gria2*, and *Mmp3*) of these estrogen-responsive genes were overexpressed in both spontaneous and DES-associated leiomyomas.

## MATERIALS AND METHODS

### Animals and Treatments

Eker rats from a closed colony at MD Anderson Cancer Center were cared for in accordance with the study protocol approved by MD Anderson Animal Care and Use Committee. Eker rats were given water and standard rat chow (Harlan Teklad 22/5 Rodent Diet) *ad libitum* and maintained on a 14:10 light–dark cycle, conditions historically associated with a 65% leiomyoma incidence in this animal model. The reprogramming effect of xenoestrogen exposure was examined after treating neonatal Eker rats on post-natal (PND) 10–12 with 1000 µg/kg/d of DES (Sigma Chemical Co., St. Louis, Mo) in sesame oil, using a total of 50 µL of sesame oil vehicle per injection. A separate group of animals were given 50 µL of sesame oil injections as vehicle (VEH) controls. The dose of DES chosen for this study was derived from earlier studies in CD-1 mice, which demonstrated similar reproductive tract abnormalities to those seen in women exposed to DES.<sup>21</sup> For microarray analysis, 16-month-old Eker rats were sacrificed for tumor and myometrium collection. For developmental reprogramming analysis, 5-month-old animals were sacrificed for myometrium collection to determine reproductive tract morphology and to isolate ribonucleic acid (RNA) for gene expression analysis. Analysis of estrogen response in neonatal uteri was evaluated by sacrificing PND 12 animals 6 hours after the last of 3 DES or VEH injections.

### Tissue Collection and Histology

Tissues (tumors and whole neonatal uteri or adult myometrium) were snap frozen in liquid N<sub>2</sub> or fixed in 10% neutral buffered formalin (NBF) for 24 to 48 hours and then transferred into 70% ethanol before paraffin embedding, sectioning, and staining for immunohistochemistry. For gene expression analysis, uteri from adult animals were scraped with a sterile scalpel in cold phosphate buffered saline (PBS) to obtain myometrium free of

endometrium, and along with tumor tissue, snap frozen in liquid N<sub>2</sub> and stored at -80°C. To obtain adequate amounts of tissue for RNA extraction from neonatal animals, 3 uteri were pooled together per sample. Cardiac blood was also taken from 5- and 16-month-old animals and allowed to clot overnight at 4°C then serum was separated by centrifugation at 4°C and stored at -20°C until analyzed for hormone (E<sub>2</sub> and P<sub>4</sub>) levels.

### Histological Staging of Estrus

Staging was performed in accordance with the procedure described by Cook et al.<sup>19</sup> Briefly, the stage of estrus or reproductive senescence was determined by histological examination of ovary, vagina, and uterine tissues of 5- and 16-month-old rats. For 16-month-old rats, they were classified by stage of reproductive senescence (pseudo pregnant [PP], persistent estrus [PE], or anestrus [AN]). For 5-month-old rats, stage of estrus was categorized into proliferative (proestrus [Pro] and estrus [E]) or secretory (metestrus [Met] and diestrus [Di]) phases. For DES-treated rats that do not cycle normally due to disrupted ovarian function,<sup>19</sup> estrogen and progesterone levels were determined via radioimmunoassay (RIA) obtained using the Ultra-sensitive Estradiol and Active Progesterone kits from Diagnostic Systems Laboratories (Webster, Tex) according to the protocol described by Cook et al.<sup>19</sup> After RIA analysis, DES-treated rats were grouped into either high estrogen and progesterone levels (High E/P) and compared with proliferative phase animals, or low estrogen and progesterone (Low E/P) for comparison with secretory phase animals.

### Microarray Analysis

Ribonucleic acid from tumors and normal myometrium of 16-month-old Eker rats was isolated as described previously<sup>20</sup> and 10 µg of cRNA fragmented and hybridized to an Affymetrix Chip Rat 230 2.0 array according to manufacturer's protocol (Affymetrix, Santa Clara, Calif). Analysis of the array was conducted using the GCOS (Affymetrix GeneChip Operating Software) program with confidence interval set at 95% and differences in fold change in gene expression were significant at  $P < .05$ .

### Microarray and Statistical Analysis

For analysis of the gene expression changes from the Gene Chip Rat Genome 230 2.0 array, raw signal intensities for each probe, set as they are contained in the

CEL files, were analyzed using Chen's dChip (<http://biosun1.harvard.edu/complab/dchip/>) model-based expression analysis to obtain significant genes with fold changes of >1.5 or <-1.5, and a false discovery rate <0.4% of 50 permutations. After obtaining model-based expression values, hierarchical clustering high-level analysis was performed using dChip. For statistical analysis of real-time PCR data, a linear model analysis was applied to estimate the interaction effects of gene and treatment.

### Microarray Validation and Real-time PCR

Validation of microarray data was conducted by real-time PCR of complementary deoxyribonucleic acid (cDNA) from identical samples (if available) or comparable age-matched and stage-matched myometrium and tumors. Frozen tissue (tumors or myometrium) from 16-month-old animals were crushed under liquid N<sub>2</sub> with mortar and pestle and RNA isolated after DNA removal according to protocol using Ambion's RiboPure Kit (Austin, Tex). Following RNA extraction, cDNA was made by reverse-transcribing 1 µg of RNA using the Invitrogen Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, Calif). Aliquots of cDNA were made for each sample and stored at -20°C until analyzed. Real-time PCR was performed using the 7900T Fast Real-Time detection system from Applied Biosystems (ABI, Foster City, Calif). Fast Real-Time Taq-Man assays from ABI were used to analyze gene expression of *Gdf10*, *Cspg2*, *Car8*, *Calbindin D9k*, *Vcam1*, *Kcnf1*, *Rasd2*, *Sfrp2*, *Tacstd1*, *Krt19*, *Rps6kb1*, *Nr2f2*, *Gria2*, *Igfbp5*, *Spp1*, *Dio2*, *Aqp3*, *Ramp3*, and *Mmp3*. All real-time PCR reactions were performed by mixing Universal Fast Real-Time Master Mix from ABI together with the gene assay mix first and then adding 2 µL of cDNA from each sample to make up a 25 µL volume. For an endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used, which included probe and forward and reverse primer in a 25 µL reaction volume. The following set of conditions were used for each real-time reaction: 95°C for 10 minutes followed by 40 cycles of 1 second at 95°C and 20 seconds at 60°C. The real-time PCR reactions were all performed in triplicate and were quantified using the  $\Delta\Delta C_t$  method, which uses the average  $C_t$  of the GAPDH subtracted from the target gene  $C_t$  to obtain the average  $\Delta C_t$ . A calibrator from each set of samples was chosen from which to subtract individual VEH and DES samples  $\Delta C_t$  values to obtain the  $\Delta\Delta C_t$ . The fold change for each sample was calculated in comparison to the calibrator by taking  $2^{\Delta\Delta C_t}$ . The

calibrator for both 16-month and PND 12 rats was VEH-treated myometrium or uteri. For samples from 5-month-old rats, estrus-staged VEH-treated myometrium was used as the calibrator.

## Bioinformatics Data Analysis

To identify candidate ER target genes from the microarray analysis, we employed the use of the computational program DRAGON ERE finder (version 2.0). The program, which uses a detection algorithm formulated from the palindromic half sites of the ERE, was used to scan for putative EREs in 171 sequences identified from the microarray analysis. Estrogen response element detection was set at 83% sensitivity for analysis. (<http://sdmc.lit.org.sg/ERE-V2/index>).

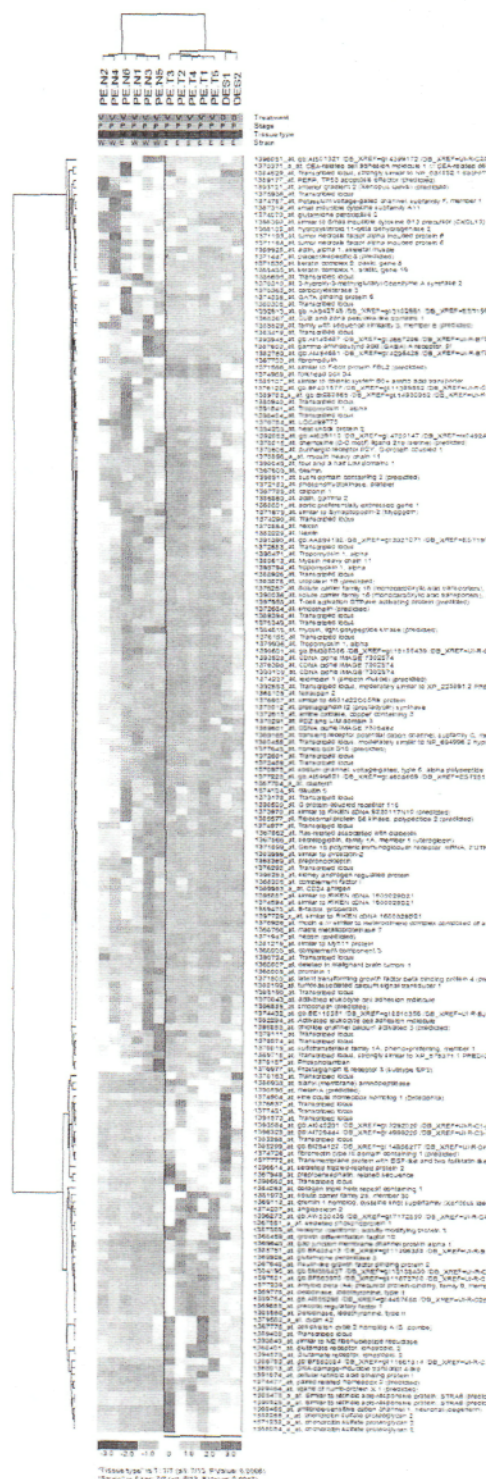
## RESULTS

## Identification of Candidate Genes for Developmental Reprogramming by DES

To identify leiomyoma-associated genes that were candidates for developmental reprogramming in the myometrium, initially we conducted microarray analysis of age-matched and stage-matched myometrium and uterine leiomyomas from Eker rats. Tissues (tumor or normal myometrium) were obtained from rats at 16 months of age (the age at which 65% of Eker rats develop uterine leiomyomas) and classified as 1 of the 3 stages of reproductive senescence PP, PE, or AN (as described in the Materials and Methods). Age-matched PE myometrium (n = 6) and PE tumors (n = 7) from rats were analyzed using the Affymetrix Rat 230 2.0 array.

Microarray analysis of age-matched and stage-matched myometrium and leiomyomas identified a total of 171 differentially expressed genes. Unsupervised hierarchical clustering clearly distinguished normal myometrium from tumors (Figure 1). Two of the tumors were from neonatally DES-exposed rats, which formed a distinct node within the cluster that contained the other 5 spontaneous tumors from untreated animals, indicating that their expression profile had characteristics both shared and distinct from tumors that arose in animals that had not been exposed to DES (Figure 1).

We have previously demonstrated that expression of *Pgr* and *Calbindin D9k*, 2 estrogen-regulated genes



**Figure 1.** Representative heat map of microarray analysis. Heat map represents unsupervised comparison of VEH PE myometrium and DES PE tumors from 16-month-old animals; VEH = vehicle; DES = diethylstilbestrol; PE = persistent estrus; N = normal myometrium; T = tumor.

**Table 1.** Identification of Predicted or Functional Estrogen Response Elements (EREs)

Gene	Predicted ERE (Forward)	Predicted ERE (Reverse)	ERE Location Relative to the 5' End of the Gene
<i>Dio2</i>		GT-GGTCA-TCT-CAACC-CT	R = -9376
<i>Calbindin D9k</i>	CA-GGTCA-GGG-TGATC-TT	AA-GATCA-CCC-TGACC-TG	F = 50; R = -66
	AG-GGTCT-ATG-TAGCC-CA		F = 1507
<i>Gria2</i>	GA-GGTCA-GTC-TGATC-AA	GG-GGGCA-TTG-TGACC-CT	F = 19005; R = -51017
	AA-AATCA-AAT-TGACC-CC	CC-AGTCA-TTC-TGCCC-AA	F = 19719; R = -2244
<i>Car8</i>	GT-AGTCA-CAC-CAACC-CT	TG-GGTCC-ACT-TGCCC-TC	F = 33744; R = -38190
	CA-AGTCA-AAA-CAACC-CT	AA-GGTGG-CTT-TGTCC-AG	F = 45678; R = -9263
<i>Gdf10</i>		AT-GGACA-ATA-TGACC-TT	R = -10563
		AT-GGTCA-AGA-TGCCT-GA	R = -10442
		AG-GCTCA-CAC-TGCCC-TC	R = -7249
<i>Mmp3</i>		GT-AGTCA-CCA-CACCC-TT	R = -12519

The Dragon ERE program ver.2.0 was used to identify predicted EREs using 0.83 sensitivity. Predicted ERE sequences, forward and reverse, are listed for each gene.

expressed in the myometrium, become developmentally reprogrammed in the adult myometrium as a result of neonatal DES exposure.<sup>19</sup> To identify estrogen-responsive genes associated with the development of leiomyomas that could be candidates for developmental reprogramming, we conducted an *in silico* analysis of these 171 differentially expressed genes using the DRAGON ERE finder program to determine if they contained a functional or putative ERE. Table 1 illustrates the result of this analysis for 6 representative genes. We identified 26 genes that had known functional EREs and 86 that had putative EREs. Thus, of the 171 differentially expressed genes, 112 (65%) were potentially regulated by estrogen, consistent with the hypothesis that hormones, especially estrogen, contribute to the development of uterine leiomyoma. From these, we selected a subset of 19 genes, based on biological plausibility (Table 2) and used real-time PCR analysis to validate our microarray data. Real time RT-PCR analysis confirmed differential expression in tumor versus normal myometrium of 18 out of these 19 genes (Table 2), and these 18 genes were then evaluated as candidates for developmental reprogramming in the myometrium of DES-exposed rats.

### Identification of Genes Developmentally Reprogrammed by Neonatal DES Exposure

For this analysis, we defined a developmentally reprogrammed gene as one that was estrogen responsive and displayed an altered expression pattern in adult myometrium of rats exposed neonatally to DES. Myometrium

**Table 2.** Microarray Gene Validation<sup>a</sup>

Gene	FC-Array	FC-Real-Time PCR
<i>Gdf10</i>	6.73	19.97
<i>Cspg2</i>	3.33	1.99
<i>Car8</i>	4.34	2.04
<i>Calbindin D9k</i>	9.63	28.05
<i>Vcam1</i>	2.31	-1.25
<i>Kcnf1</i>	2.51	1.74
<i>Rasd2</i>	4.77	11.47
<i>Gria2</i>	37.02	17.53
<i>Dio2</i>	5.23	3.43
<i>Mmp3</i>	5.32	38.20
<i>Sfrp2</i>	6.57	7.06
<i>Tacstd1</i>	-12.87	-177.30
<i>Krt19</i>	-12.63	-440.20
<i>Rps6kb1</i>	-6.36	-2.07
<i>Nr2f2</i>	-3.96	-2.11
<i>Igf1bp5</i>	-3.32	-8.65
<i>Spp1</i>	-3.24	-1.46
<i>Aqp3</i>	-4.08	-16.39
<i>Ramp3</i>	-5.53	-11.31

Abbreviations: FC, fold change.

<sup>a</sup> Validation of 19 candidate genes using real-time quantitative RT-PCR. Data are a representative of two independent measurements with samples run in triplicate. Fold change (FC) is based on the change in fold induction (calibrator sample set at 1) of the diethylstilbestrol (DES) group as compared to the vehicle (VEH) groups with samples normalized to GAPDH expression.

from 5-month-old adult rats neonatally exposed to DES were divided into high estrogen and progesterone (High E/P) and low estrogen and progesterone (Low E/P) groups and compared to myometrium from VEH grouped into proliferative (estrus and proestrus) and

**Table 3.** Gene Expression (Vehicle [VEH] versus Diethylstilbestrol [DES]) in Neonatal Uteri<sup>a</sup>

Gene	Normal Estrogen Response	Neonatal DES Response	
		Fold Change	Standard Deviation
<i>Calbindin D9k</i>	Induced	39.12	1.53
<i>Dio2</i>	Induced	7.31	0.19
<i>Gdf10</i>	Repressed	-4.78	0.06
<i>Car8</i>	Repressed	-7.14	0.05
<i>Gria2</i>	Repressed	-83.33	0.03
<i>Mmp3</i>	Repressed	-3.89	0.04

<sup>a</sup> Gene expression in neonatal uteri treated with VEH or DES. Fold change relative to VEH control. Vehicle values set to 1 with decreases indicated by negative values with all samples normalized to GAPDH expression. Data are mean  $\pm$  SD of two independent measurements with samples run in triplicate.

secretory (metestrus and diestrus) phases, respectively, for the analysis of developmental programming.

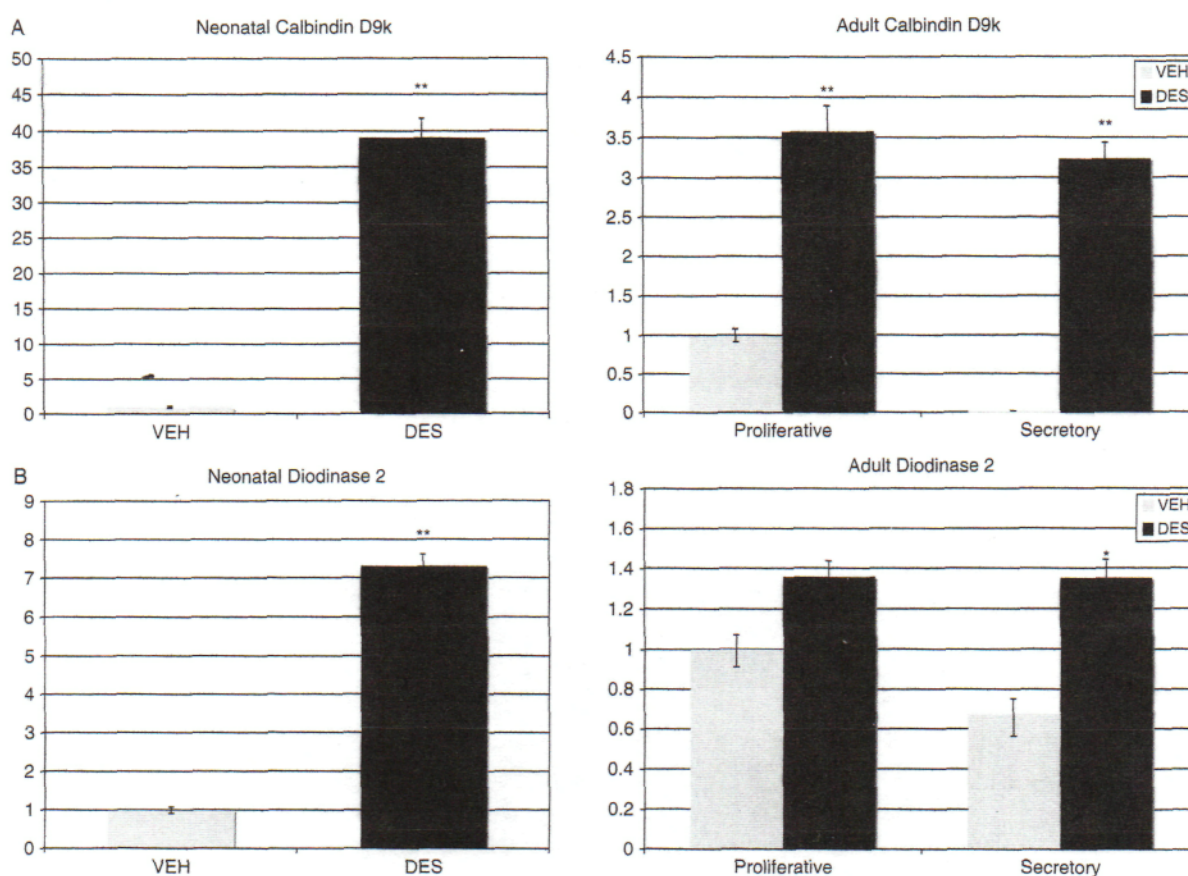
Initially, the estrogen responsiveness of candidate genes was confirmed in neonatal uteri, where gene expression was demonstrated to be either repressed or induced in response to DES (Table 3). As shown in Figure 2, some genes were induced (illustrated by *Calbindin D9k*, and *Dio2*) by hormone and others were repressed (illustrated by *Gdf10*, *Car8*, *Gria2*, and *Mmp3*; Figure 3) in neonatal uteri. Furthermore, this pattern of estrogen responsiveness (induced vs. repressed) was confirmed by analyzing the expression of these genes during the normal estrus cycle in adult myometrium, where the expression pattern during the estrus cycle was concordant (ie induced or repressed) with their response to DES in neonatal uteri (Figures 2 and 3). Inducible genes were upregulated during the proliferative (high estrogen) versus secretory (low estrogen) phase of the normal estrus cycle whereas repressed genes were downregulated in proliferative versus secretory phases. Once the estrogen responsiveness of the 18 candidate genes was confirmed, we next determined if expression of these genes had been reprogrammed in the adult myometrium of rats exposed neonatally to DES. As shown in Table 4, 6/18 of these estrogen-responsive genes were developmentally reprogrammed by neonatal exposure to DES.

*Calbindin D9k*, which we had previously characterized as a gene developmentally reprogrammed by DES,<sup>20</sup> was also identified in this analysis as being reprogrammed in the adult myometrium of neonatally DES-exposed animals

(Figure 2), serving as an internal positive control for the microarray-based approach. Similar to *Calbindin D9k*, *Dio2*, another myometrial estrogen-inducible gene, also became overexpressed in the adult myometrium, as evidenced by elevated expression in myometrium of 5-month-old rats during both proliferative and secretory phases (Figure 2 and Table 4). Although the expression of *Dio2* in high E/P DES animals was higher but not significantly so relative to proliferative phase myometrium (when the gene is normally induced), it was significantly higher in low E/P DES animals relative to normal secretory phase myometrium. This pattern was also seen for *Calbindin D9k*, where differential gene expression was even more pronounced in low E/P DES animals relative to normal secretory phase myometrium. Thus *Dio2* displayed a characteristic DES-induced hyper-responsiveness to even low levels of estrogen, as previously reported for *Calbindin D9k*.<sup>20</sup>

Strikingly, in animals exposed neonatally to DES, the expression of several genes normally repressed by estrogen (*Gdf10*, *Car8*, *Gria2*, and *Mmp3*) was significantly increased (rather than repressed) under conditions of high estrogen (proliferative phase) relative to normal myometrium (Figure 3 and Table 4). Thus, the overall effect of developmental reprogramming by DES was to induce an increase in expression of estrogen-responsive genes, even those normally repressed by this hormone.

Because the net effect of developmental reprogramming was to elevate expression and/or induce the persistent expression of estrogen-responsive genes in the myometrium, we next asked if elevated expression of these genes was a generalized finding in tumors. Real-time PCR of normal myometrium and tumors from age-matched (16 month) and stage (PE)-matched animals revealed that 5/6 (*Gdf10*, *Car8*, *Gria2*, *Dio2*, and *Mmp3*) of the developmentally reprogrammed genes were overexpressed in tumors (n = 8) as compared to normal PE myometrium (Figure 4 and Table 5). Importantly, these genes were overexpressed in spontaneous tumors from both DES-exposed (n = 4) and nonDES-exposed (n = 4) rats. Thus, the reprogramming of gene expression in the myometrium induced by neonatal DES resulted in elevated expression of these genes in a pattern characteristic of leiomyomas. Taken together, these data are consistent with the hypothesis that increased expression of estrogen-responsive genes contributes to the development of uterine leiomyoma and that increased susceptibility for tumor development in DES-exposed animals is due to elevated expression of estrogen-responsive genes, mimicking the promotional effect of this hormone on leiomyoma development.



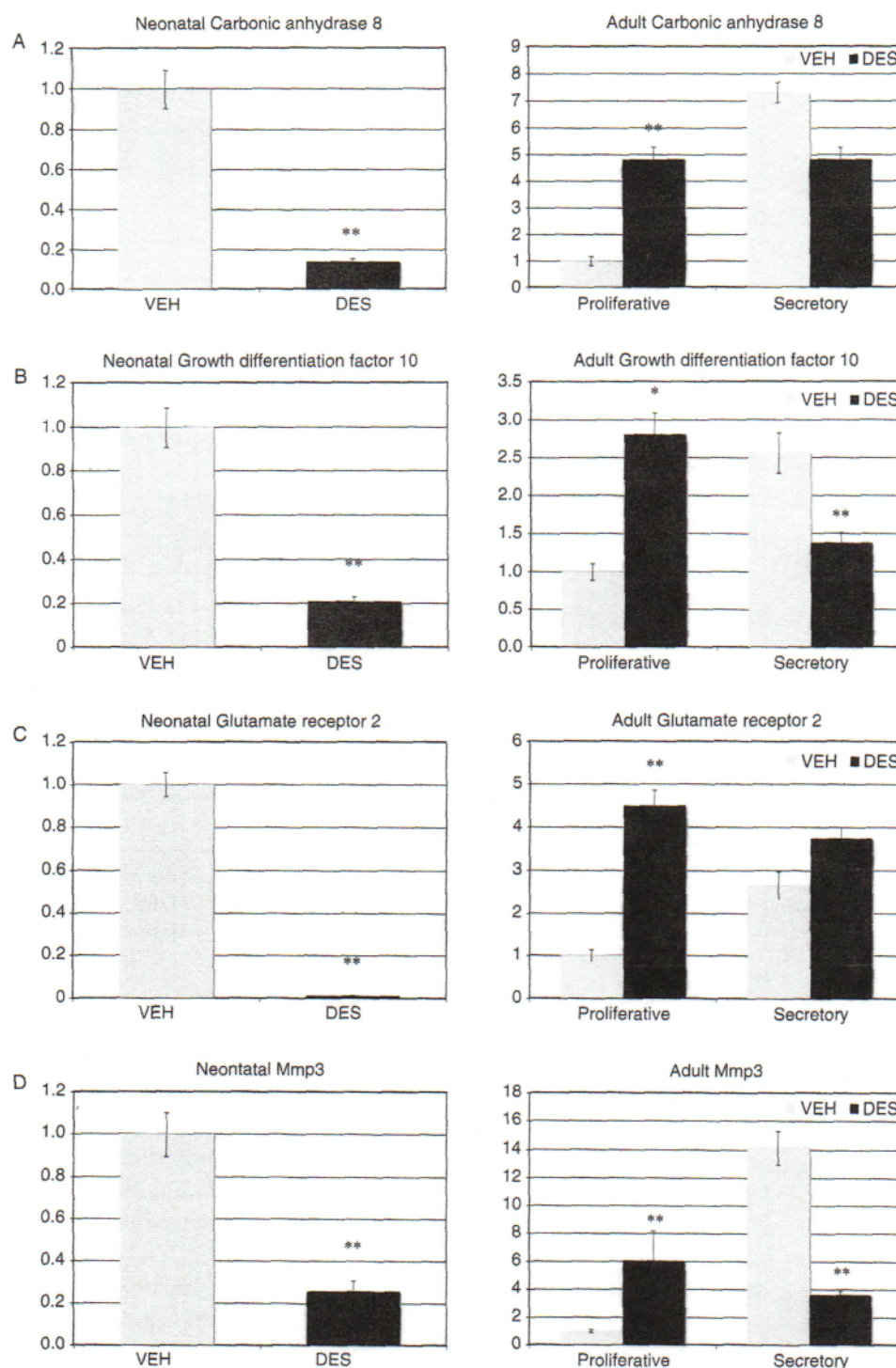
**Figure 2.** Real-time RT-PCR for DES-reprogrammed genes induced by hormone in uteri (PND 12; left panel), and myometrium (5 months; right panel). A-B, Gene expression for *Calbindin D9k* and *Diodinase 2* (*Dio2*) at PND 12 (VEH, n = 9; DES, n = 9) and 5 months (VEH = 5, DES = 5). \*\* =  $P \leq .001$ , \* =  $P \leq .05$ . DES = diethylstilbestrol; RT-PCR = Reverse transcriptase–polymerase chain reaction; VEH = vehicle.

## DISCUSSION

Diethylstilbestrol has been demonstrated in several systems to induce developmental reprogramming of the female reproductive tract at the molecular, cellular, and tissue levels, which leads to development of reproductive cinoma and uterine leiomyoma). However, the precise nature of the reprogramming events leading to tumorigenesis is not fully understood. We have previously identified a critical window of susceptibility to developmental reprogramming of the uterus (PNDs 3–12) after neonatal DES exposure,<sup>20</sup> which can increase tumor incidence to 100%. To define the mechanism by which DES reprograms the uterus to increase susceptibility to tumorigenesis, we initiated this study to identify genes in tumors that could be targets for developmental reprogramming in the myometrium. Using gene expression profiling of uterine leiomyomas and myometrium, 18 candidate estrogen-responsive genes were selected, of which, 6

(*Gdf10*, *Car8*, *Calbindin D9k*, *Gria2*, *Dio2*, and *Mmp3*) were shown to be reprogrammed in the adult myometrium by neonatal DES exposure. Elevated expression of 5 of these genes, *Gdf10*, *Car8*, *Gria2*, *Dio2*, and *Mmp3* in 8/8 tumors, including tumors that arose without DES treatment, demonstrated that DES developmentally reprogrammed estrogen-responsive genes associated with tumor development.

Similar to a previous study looking at the molecular effects of neonatal DES exposure on the endometrium,<sup>21</sup> our transcriptional profiling of myometrium and tumors revealed that more than half (112/171) of genes differentially expressed in tumors were either putative or known estrogen-responsive genes, lending support to the importance of estrogen in promoting the growth of uterine leiomyomas. Altered expression of estrogen-responsive genes has also been demonstrated in hamster and mouse whole uteri neonatally exposed to DES, which ultimately develop reproductive tract neoplasias, although the



**Figure 3.** Real-time RT-PCR for DES-reprogrammed genes repressed by hormone in utero (PND 12; left panel), and myometrium (5 months; right panel). A-D, Gene expression for *carbonic anhydrase 8* (*Car8*), *growth differentiation factor 10* (*Gdf10*), *glutamate receptor 2* (*Gria2*), and *matrix metalloproteinase 3* (*Mmp3*) at PND 12 (VEH, n = 9; DES, n = 9), and 5 months (VEH = 5, DES = 5). \*\* =  $P \leq .001$ , \* =  $P \leq .05$ . DES = diethylstilbestrol; RT-PCR = Reverse transcriptase–polymerase chain reaction; VEH = vehicle.

myometrium and the genes identified in our analysis were not specifically investigated in those studies.<sup>22,23</sup> Furthermore, in our study, the reprogramming of

estrogen-responsive genes occurred in the myometrium of neonatally DES-exposed animals before the onset of tumors. This suggests that DES increases tumor incidence

**Table 4.** Gene Expression in Myometrium of Diethylstilbestrol-exposed Rats

Gene	Normal Estrogen Response	Adult Estrogen Response	
		Fold Change (relative to VEH)	Standard Deviation
<i>Calbindin D9k</i>	Induced	3.40	0.16
<i>Dio2</i>	Induced	2.00	0.12
<i>Gdf10</i>	Repressed	2.91	0.25
<i>Car8</i>	Repressed	4.83	0.17
<i>Gria2</i>	Repressed	4.20	0.15
<i>Mmp3</i>	Repressed	6.10	0.18

<sup>a</sup> Gene expression in myometrium of 5-month-old diethylstilbestrol-exposed rats. Fold change relative to vehicle (VEH) control. Vehicle values set to 1 with all samples normalized to GAPDH expression. Data are mean  $\pm$  SD of two independent measurements with samples run in triplicate.

by increasing the expression of estrogen-responsive genes, possibly mimicking the promotional effect of estrogen during spontaneous tumor development. Along with previous studies, our data identify altered expression of estrogen-responsive genes as a hallmark of xenoestrogen exposure that precedes, and likely contributes to, development of reproductive tract disease, including uterine leiomyoma.

In the rat uterus, DES both induced and repressed gene expression, similar to what has been seen for estrogens in other estrogen-responsive tissues.<sup>24,25</sup> We observed that neonatal DES exposure increased the expression of genes that were normally induced by estrogen and led to persistent expression under conditions when estrogen levels are normally insufficient to drive gene expression (ie, low estrogen levels or secretory phase), consistent with these genes having become hyper-responsive to endogenous estrogen. Importantly, we also found that several genes normally repressed by estrogen exhibited elevated (rather than decreased) expression under conditions when estrogen levels were high. Thus, reprogramming by DES caused both quantitative and qualitative changes in the way reprogrammed genes responded to estrogen.

Several mechanisms for altered estrogen responsiveness as a result of developmental reprogramming have been investigated in other tissues. den Hollander and colleagues<sup>26</sup> proposed that one mechanism for estrogen hyper-responsiveness occurs via epigenetic reprogramming of the estrogen receptor co-activator, Ciz1, which amplifies estrogen responsiveness in breast cancer cells

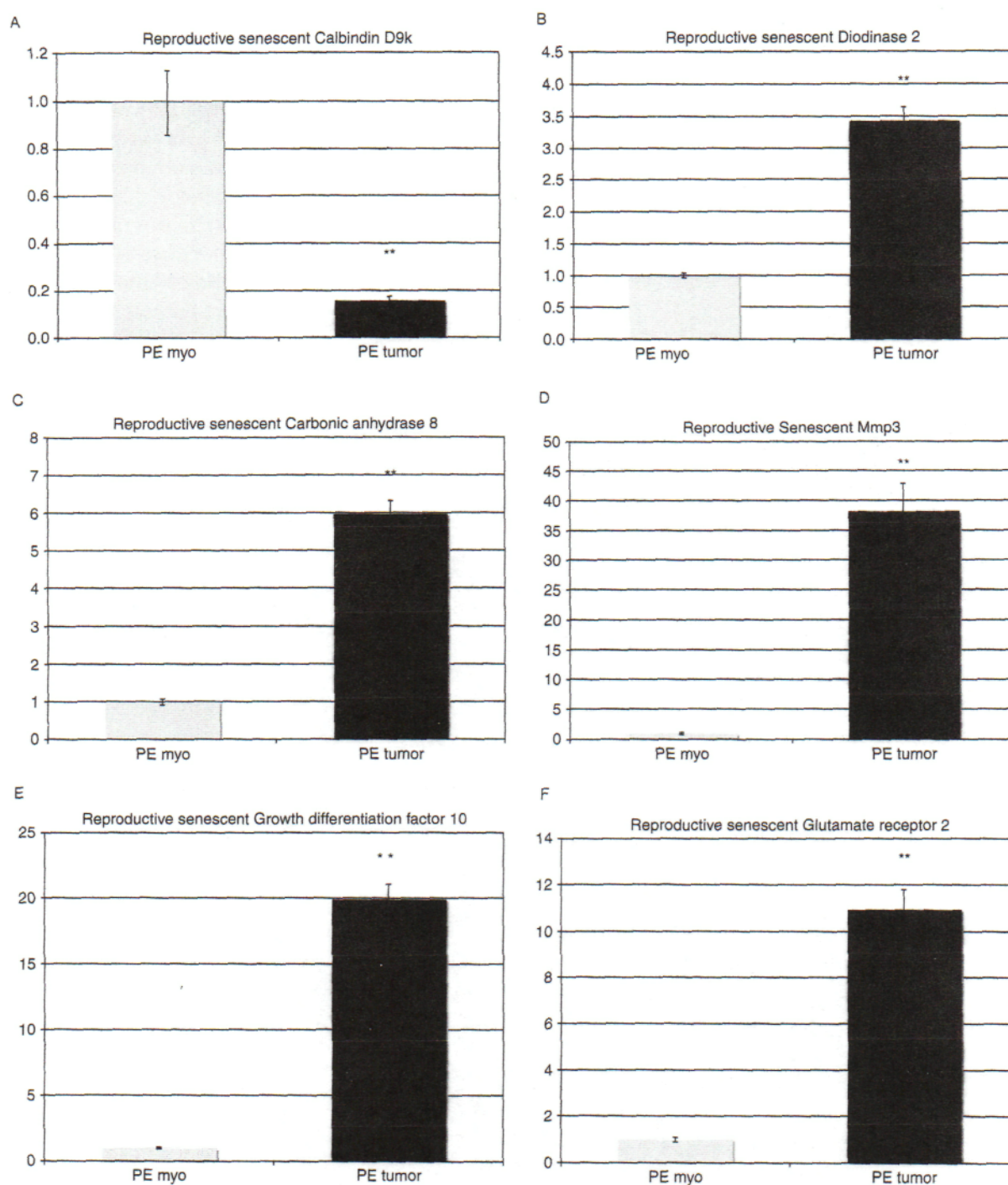
by increased recruitment of the ER complex to chromatin of target genes. A direct epigenetic mechanism to maintain hormonal "memory" at sites of specific genes through DNA methylation has also been described by Thomassin and colleagues<sup>27</sup> via alterations in DNA methylation of the tyrosine aminotransferase (*Tat*) gene after fetal exposure to glucocorticoids. Similarly, differential DNA methylation of the lactoferrin gene promoter and phosphodiesterase type 4 variant 4 (*PDE4D4*) gene has also been observed in response to neonatal xenoestrogen exposure in the uterus and prostate, respectively.<sup>28,29</sup> In the prostate, these changes occur very early, with hypomethylation of *PDE4D4* observed before the formation of prostatic neoplastic lesions (PIN) in neonatally xenoestrogen-exposed mice. Both studies demonstrate that neonatal exposure to xenoestrogen can induce hypomethylation of specific genes, which is maintained in the neoplastic lesions that arise in these tissues (uterine tumors and PIN). This suggests that gene hypomethylation induced by xenoestrogens can serve as a biomarker of exposure before neoplasias are observed. We also observed developmental reprogramming in the "normal" myometrium at 5–6 months, prior to tumorigenesis in exposed females. Although the exact mechanism(s) responsible for developmental reprogramming of gene expression in the myometrium have not been identified, these studies lend support to the hypothesis that a xenoestrogen "fingerprint," such as a specific DNA methylation event, or altered expression of a group of developmentally reprogrammed estrogen-responsive genes, could be used as biomarkers to help identify individuals exposed neonatally to xenoestrogens who may be susceptible to adult disease, including uterine leiomyoma.

### Ontology of Developmentally Reprogrammed Genes

Given the potential for developmental reprogramming of gene expression to contribute to tumorigenesis, a gene ontology was compiled for the genes identified in this study that were developmentally reprogrammed by neonatal DES exposure, in the context of the potential involvement of these genes in the human disease.

### Cell Signaling: *Car8* and *Gria2*

*Car8*, which is developmentally regulated, and *Gria2*, which is gestationally regulated, were both developmentally reprogrammed and overexpressed in tumors. The



**Figure 4.** Real-time RT-PCR for reprogrammed genes differentially expressed in PE tumors versus PE myometrium from 16-month Eker rats. A-F, Gene expression for *Calbindin D9k*, *deiodinase 2 (Dio2)*, *carbonic anhydrase 8 (Car8)*, *matrix metalloprotease 3 (Mmp3)*, *growth differentiation factor (Gdf10)*, and *glutamate receptor 2 (Gria2)*; PE myometrium, n = 2; PE tumors, n = 8) \*\* =  $P \leq .001$ , \* =  $P \leq .05$ . RT-PCR = Reverse transcriptase–polymerase chain reaction; PE = persistent estrus.

function of *Car8*, part of the acatalytic carbonic anhydrase-related protein family (CARP), remains unknown; however, it has been shown to play a role in fetal brain development<sup>30,31</sup> as well as in the promotion

of colon cancer via increased cell proliferation and invasion.<sup>32</sup> Additionally, *Gria2*, a receptor that functions as an alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)–sensitive neurotransmitter, has

**Table 5.** Gene Expression in Tumors Versus Normal Myometrium<sup>a</sup>

Gene	Tumor Response	
	Fold Change	Standard Deviation
<i>Calbindin D9k</i>	-6.25	0.07
<i>Dio2</i>	3.43	0.12
<i>Gdf10</i>	19.97	0.6
<i>Car8</i>	5.98	0.21
<i>Gria2</i>	11.00	0.34
<i>Mmp3</i>	38.32	3.59

<sup>a</sup> Gene Expression in normal persistent estrus (PE) myometrium and PE tumors (Vehicle (VEH) and diethylstilbestrol (DES)). Fold change relative to normal PE myometrium. Normal PE myometrium values set to 1 with decreases indicated by negative values and all samples normalized to GAPDH expression. Data are mean  $\pm$  SD of two independent measurements with samples run in triplicate.

garnered significant interest in the uterine leiomyoma field due to its appearance in multiple human microarray studies of uterine leiomyoma. Subsequently, Tsibris and colleagues<sup>33</sup> hypothesized that *Gria2* could participate synergistically with estradiol, retinoic acid, and its receptor RXR to support tumor growth. The reprogramming of this gene in animals at increased risk for leiomyoma and its overexpression in human leiomyomas suggest that *Gria2* is a strong candidate gene for participation in the development of this disease.

### Extra Cellular Matrix Proteins: *Mmp3*

A gestationally and neonatally regulated gene<sup>34</sup> that is necessary for tissue development and wound healing,<sup>35</sup> *Mmp3* was developmentally reprogrammed to become induced rather than repressed under high estrogen conditions. This reprogramming was observed before the onset of tumors, and like *Car8* and *Gria2*, *Mmp3* was also elevated in tumors. It is well accepted that one of the main driving forces in the growth of uterine leiomyomas is increased expression of extracellular matrix proteins (ECM). It is not surprising, therefore, that *Mmp3*, which is involved in ECM remodeling, was also overexpressed in uterine leiomyomas. However, the fact that this gene was reprogrammed prior to the development of tumors suggests that increased expression of *Mmp3* could be participating in tumorigenesis, rather than a consequence/epiphenomena associated with the increased matrix production characteristic of these tumors.

### Thyroid Hormone Metabolism: *Dio2*

A gene involved in thyroid hormone metabolism, *Dio2*, also exhibited elevated expression in the myometrium of rats exposed neonatally to DES, with elevated expression also observed in tumors. *Dio2*, which converts T3 to T4 after activation by ERK 1/2, is necessary to regulate thyroid hormone levels during development, particularly in the brain,<sup>36</sup> and during pregnancy.<sup>37</sup> Specifically, *Dio2* has been identified as an estrogen-responsive gene due to elevated expression during proestrus as compared to diestrus.<sup>38</sup> Our data also indicate that *Dio2* responds to estrogen in the rat myometrium, and importantly, that *Dio2* appears to become hyper-responsive to estrogen as a result of developmental reprogramming, with elevated expression observed under both high and low estrogen conditions. Interestingly, several other reports have implicated *Dio2* in papillary thyroid cancer,<sup>39</sup> follicular carcinomas,<sup>40</sup> and mesotheliomas,<sup>41</sup> suggesting that *Dio2* could be associated with tumors of organs other than the thyroid, although, a mechanism by which *Dio2* could participate in uterine leiomyoma is unknown. However, given that mitogens such as insulin, EGF, and IGF-1 (which are overexpressed in leiomyomas) also induce *Dio2* mRNA,<sup>36,42</sup> it is unclear at this time if expression of *Dio2* in tumors is due to altered expression of these growth factors or reflective of the hyper-responsiveness to estrogen that is characteristic of these tumors.

### Growth Factors: *Gdf10*

*Gdf10* is expressed in the normal adult uterus<sup>43</sup> and has been shown to regulate growth during development, mainly head development.<sup>44</sup> *Gdf10* (*Bmp3b*) is also a member of the transforming growth factor (TGF) $\beta$  super family, which is involved in regulating cell differentiation, proliferation, and apoptosis.<sup>43</sup> In addition, *Gdf10* is characterized as target of the canonical Wnt pathway,<sup>45</sup> specifically in the development of neural tissue. *Gdf10* is normally repressed by estrogen; however, in rats exposed neonatally to DES, it was overexpressed in the adult myometrium when estrogen was high, and was expressed at higher levels in tumors relative to age-matched and stage-matched myometrium. Interestingly, several studies observed that the *Gdf10* gene is hypermethylated in both human bladder tumors and cells collected from the urine of bladder cancer patients.<sup>46</sup> The CpG island in the *Gdf10* gene is also hypermethylated in non-small cell lung carcinomas (NSCLC), indicating that *Gdf10* can be epigenetically regulated in some cancers.<sup>47</sup>

Given that TGF $\beta$  signaling promotes mesenchymal cell growth, it is interesting to speculate that increased signaling via this pathway as a result of elevated *Gdf10* expression may be contributing to leiomyoma growth. Since TGF $\beta$  signaling is known to contribute to leiomyoma growth,<sup>48</sup> further study of the role of *Gdf10* in development of these tumors is warranted.

### Calcium Ion Binding Proteins: *Calbindin D9k*

In addition to *Mmp3*, another calcium ion binding protein *Calbindin D9k* is developmentally reprogrammed by xenoestrogen exposure. *Calbindin D9k* has multiple biological roles including fetal calcium uptake and uterine contractions,<sup>49</sup> calcification of bone and teeth, and intestinal calcium uptake and transport.<sup>50</sup> Importantly, it is overexpressed in the immature uterus after exposure to endocrine disruptors,<sup>51-53</sup> as well as in the adult uterus during pregnancy and proestrus.<sup>50,54</sup> This suggests that *Calbindin D9k* is an ideal biomarker for estrogen exposure in the myometrium, and as shown in our studies, an excellent reporter of developmental reprogramming by xenoestrogens in this tissue.

In summary, these data demonstrate that neonatal exposure to DES, and perhaps other xenoestrogens as well, can modulate the expression of the estrogen-responsive genes associated with uterine leiomyomagenesis. Several of these genes have also been identified as overexpressed in human leiomyomas, further suggesting that they may be key targets for early life exposures to xenoestrogens. The identification of genes that participate in uterine leiomyoma development continues to be a critical need; both to better understand the etiology of these tumors and for the development of new therapeutic approaches for this disease. In particular, with the identification of genes susceptible to developmental reprogramming in the myometrium, in the future it will be possible to identify causal mechanism(s) of developmental reprogramming in this tissue, and enhance our understanding of fundamental processes that link environmental exposures to development of this disease.

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## APPENDIX B: METHODOLOGY FOR ULTRADEEP BISULFITE SEQUENCING

## **Methodology for Ultradeep Bisulfite Sequencing**

### *Animals and Treatments*

As described previously in chapter 3, Eker rats bred from our colony were utilized for this study in accordance with the guidelines of the M. D. Anderson Cancer Center Animal Care and Use Committee. Female Eker rats were fed standard rat chow (Harlan Teklad 22/5 rodent Diet) and water *ad libitum* and maintained on a 14:10 light-dark cycle. The differential DNA methylation effects of DES were analyzed after exposing neonatal Eker rats on postnatal days 10 through 12 with DES (1mg/kg) (Sigma Chemical Co., St. Louis, MO) dissolved in sesame oil or to VEH (sesame oil) control using a total of 50  $\mu$ l of DES or VEH for each subcutaneous injection, as describe in chapter 3. Animals (DES n=24, VEH n=25) were aged out to 5 months and were sacrificed at this age via CO<sub>2</sub> asphyxiation. Uteri were harvested and scraped with a sterile scalpel in cold PBS to remove endometrium from myometrium, immediately frozen in liquid nitrogen and stored at -80°C for later DNA methylation analysis. Another group of animals, age 16 months, were utilized in order to obtain unexposed normal senescent myometrium or tumors from animals exposed to VEH, as describe in chapter II and III. In addition, blood was collected from 5-month-old animals in order to obtain serum for RIA analysis and staging, as described in chapter II.

### *Histology, Radioimmunoassay and Staging of Estrus*

For reproductive tract morphology analysis and estrus staging of VEH or reproductively senescent uteri, a portion of the uterine horn was fixed in 10% neutral buffered formalin and transferred to 70% ethanol after 24 hrs. Fixed uteri from female rats sacrificed at 5 or 16 months, were embedded in paraffin and stained with hematoxylin-eosin (H&E) as described previously in chapter II and III. H&E stained uteri from DES and VEH exposed animals were examined for stage of estrus (see chapter II and III methodology). Estradiol levels were determined via RIA, obtained using the Ultra-sensitive Estradiol kits from Diagnostic Systems Laboratories (Webster, TX) according to the protocol described in by the manufacturer and in chapter II. Following hormone analysis,

DES-exposed rats were grouped into either High or Low E<sub>2</sub> in order to obtain proliferative (High E) animals to compare to proliferative phase VEH animals.

#### *Extraction and Bisulfite Treatment of DNA*

For extraction of genomic DNA, a portion of frozen myometrium or tumor from adult (5 month old VEH (n=5) or DES (n=5) exposed) or aged (16 month old normal unexposed myometrium (n=5) or VEH exposed tumors (n=5)) animals that were age and stage (proliferative) matched was obtained using a steril scalpel and placed in an 1.5 mL Eppendorf tube. Tissue was digested in TNES buffer pH 7.5 (50 mM Tris (pH 7.5), 0.4 M NaCl, 100 mM EDTA, 0.5% SDS) and proteinase K at 55°C by shaking for 1hr. Saturated (~6M) NaCl was added and the mixture was vortexed followed by centrifugation at 14,000 rpm for 30 minutes. Supernatant was transferred to a new tube and cold absolute EtOH was added to precipitate DNA followed by centrifugation at 14,000 rpm for 25 minutes at 4°C. Supernatant was discarded and the pellet was washed with cold 70% EtOH followed by centrifugation at 14,000 rpm for 20 minutes at 4°C. Supernatant was discarded and the pellet was dried and then resuspended in sterile ddH<sub>2</sub>O and stored at 4°C.

For bisulfite conversion of DNA, which converts all unmethylated cytosine to uracil, 350 ng of sample DNA was used and treated with sodium bisulfate and desulphonated using the EZ DNA methylation or methylation Gold™ Kit (Zymo Research, Orange, CA) according to the manufactures protocol. Elution of bisulfite converted DNA was eluted into 10-15 µL of sterilized 1 X TE buffer (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0) and frozen back at -20°C.

#### *Primers Design and RT-PCR Amplification of Bisulfite Converted DNA*

Ten developmentally reprogrammed genes (*Gria2*, *Gdf10*, *Calbindin D9K*, *Mmp3*, *Spp1*, *Sfrp2*, *Nr2f2*, *Rasd2* and *Krt19*) previously identified in chapters II and III were chosen to analyze DNA methylation differences in adult and aged myometrium, as well as, tumors. Seven regulatory regions were annotated in

each gene (CpG islands, ER, PR, FoxA1, SRC-3, H3R17 and Pol II binding sites), which were obtained from the Myles Brown Laboratory at Dana-Farber Cancer Center. These genome wide binding sites were identified in MCF-7 cells treated with or without estradiol and probed with the antibody against each of the transcription factors listed above (except CpG islands). A ChIP-Chip tiling array was performed in the Brown laboratory and results from the analysis of this data was lifted to the rat genome for this study. The USCS genome browser was used to identify the CpG islands in and around developmentally reprogrammed genes in addition to the binding sites of the 7 regions of interest mentioned above. Annotation of each of these genes and their regulatory sites is located in Appendix C-1. For primer design of bisulfite treated DNA, primers were designed 25-30 nucleotides in length in regions of DNA not containing any CpGs for amplification of regions of interest between 220 to 700 bp in length. Except for *Gria2*, *Gdf10* and *Calbindin D9k*, all primers were designed using the publicly available program MethPrimer (1). A list of all gene primers, genomic location, size and amplicons is located in Appendix C-2. For the three genes listed above, which were used in cloning and sequencing analysis, nested primers were designed to reduce non-specific amplification of bisulfite treated DNA.

For amplification of bisulfite treated DNA, primers were first tested using gradient RT-PCR (47°C-57°C) on the TurboCycler Gradient 96 Machine (Stratagene, Cedar Creek, Texas). The PCR reaction was performed in two separate reactions (PCR-1 and PCR-2) in a 96 well-plate in a 25  $\mu$ L reaction using 1  $\mu$ L of bisulfite treated DNA (BS-DNA) together with master mix (5X) containing 10% MCA2 buffer (670 mM Tris pH 9, 160 mM ammonium sulfate, 1% Triton X-100, 100 mM 2-mercaptoethanol, 20 mM MgCl<sub>2</sub>), 1% bovine serum albumin, 0.5 % (0.125 mM) dNTPs (25 mM stock), 1% forward and reverse primer each (10  $\mu$ M stock), 1% Taq polymerase (5U/ $\mu$ L stock) (Applied Biosystems, Foster City, CA), 10% Cresol red (1mM cresol red, 10 mM Tris pH 8.8, 60% glycerol in water) plus 75% nuclease free water. To the first round of PCR-1 master mix was added an inhibitory oligonucleotide to prevent non-specific action of Taq polymerase at temperatures below 50°C at a concentration

of 0.012  $\mu$ M. DNA was amplified under the following conditions: 1, 4-minute denaturation cycle at 95°C; 5 cycles of 30 sec (95°C), 1 minute annealing temperature, 2 minute extension (72°C); 25 cycles, 30 sec (95°C), 1 minute annealing, 1 minute extension (72°C); 1 cycle, 4 minutes (72°C). Following PCR-2 under the same conditions, 15  $\mu$ L of PCR product was resolved on an agarose gel via gel electrophoresis and stained with ethidium bromide to visualize under UV light to determine amplicon size. Once all primers were tested and validated, all samples (adult myometrium (n=5), DES myometrium (n=5), senescent myometrium (n=5) and VEH tumors (n=5)) were amplified together in two round of PCR at the appropriate temperature determined during testing, as describe above, in a 96-well plate. PCR products were then subjected to filtration using the Montage PCR Centrifugal Filter Devices (Millipore, Billerica, MA) to purify and remove salts, unincorporated dNTPs and primers. Purified PCR products were eluted into 1 X TE buffer, quantitated and used immediately for cloning and sequencing or frozen at -20°C until pooling could be performed for all products for ultradeep bisulfite sequencing.

#### *Bisulfite Cloning and Sequencing*

Amplified and purified products from BS-DNA samples (5 individual samples pooled into one of 4 corresponding sample tubes; adult myo (AM), DES myo (DM), senescent myo (SM) or VEH tumor (VT)) were cloned in TOPO-TA cloning system (Invitrogen). Selection media (Kan<sup>+</sup>) was used to grow up positive colonies overnight (12-16 hrs) at 37°C. 2 $\mu$ L of colony broth was used in a PCR reaction to screen for plasmids positive for insert according the reaction described above. Plasmids positive for insert were then isolated using the GenElute™ Plasmid Miniprep kit (Sigma, St. Louis, MO). Isolated plasmid DNA was then sequenced. Between 12-44 clones from DES or VEH samples were sequenced for later analysis.

#### *Bisulfite Solexa Sequencing*

For solexa sequencing, purified PCR products were pooled in to one of four corresponding tubes (AM, DM, SM or VT) in equal molar amounts (30 ng/ $\mu$ L). Prior to sequencing, pooled PCR products were sonicated in glycerol to generate <200 bp products. cDNA libraries were created from products and sequencing tags were applied. Tagged sequences were then bisulfite sequenced on the Illumina Solexa Genome Analyzer II platform using single-end reads.

#### *Alignment and Mapping of Amplicons*

Computational analysis of the bisulfite Solexa sequencing (Bisulfite-Seq), was accomplished using BSMAP whole genome bisulfite sequence mapping program (2). Prior to mapping, both strands of the genome and solexa reads were bisulfite transformed *in silico*, changing cytosines (C) thymines (T), in order to map reads to the genome. Each converted sequence was mapped to one of 70 genome positions according to tags and primers. Detailed mapping alignment technique and algorithms are described by Xi et al. (2). Analysis of cloning and sequencing was performed using the *in silico* transformed DNA regions analogous to the amplicon to identify differences in methylation of C between the two sequences for DES or VEH samples.

#### *Statistical Analysis*

For analysis of differential DNA methylation of sequences from cloned PCR products, a mean was obtained for all methylated vs. unmethylated CpGs and % methylation was obtained based on the number of clones analyzed for each sample. A Student's t-test was applied to these data to determine if a significant difference existed in % DNA methylation between samples, a value of  $p < 0.05$  was determined to be significant. The same analysis was applied also to individual CpG sites to determine significance.

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## VITA

Kristen Leigh Greathouse was born in Fort Worth, Texas on the 22<sup>nd</sup> day of January, 1975, to Jan Davis and Wendell Van Hook. Leigh and her parents subsequently lived in several different areas of the world including Scotland, Germany, Italy, Greece, Iraq, Kuwait, as well as, in the U.S. including Oklahoma and Texas. Leigh graduated with honors in May of 1993 from Nacogdoches High School in Nacogdoches, TX and later that year started her undergraduate education at Stephen F. Austin State University in Nacogdoches, TX. Leigh received the degree of Bachelor of Science in Food Science and Nutrition in May of 1997. Upon obtaining her B.S., Leigh entered the graduate school at Texas Women's University in Denton, TX in June of 1998. In August of 2001 she graduated with her Masters of Science in Exercise and Sports Nutrition, as well as, obtained her registration as a dietitian. Leigh began her career at the Graduate School of Biomedical Sciences at the University of Texas at Houston in January of 2005. She pursued her Ph.D. in the laboratory of Dr. Cheryl Walker at the UT MD Anderson Cancer Center, Science Park Research Division, Department of Molecular Carcinogenesis in Smithville, Texas.

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