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REPRODUCTIVE AND GENOMIC EFFECTS OF GESTATIONAL AND LACTATIONAL EXPOSURE TO ESTROGENIC ENDOCRINE DISRUPTORS IN MALE MICE

By

Mark Raymond Fielden

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
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DOCTOR OF PHILOSOPHY

Department of Biochemistry and Molecular Biology

2002

ABSTRACT

REPRODUCTIVE AND GENOMIC EFFECTS OF GESTATIONAL AND LACTATIONAL EXPOSURE TO ESTROGENIC ENDOCRINE DISRUPTORS IN MALE MICE

By

Mark Raymond Fielden

There is extensive concern and debate regarding the degree to which human populations are being adversely affected from exposure to estrogenic endocrine disrupting (EED) chemicals in the environment. It has been proposed that the reported increase in the incidence of human reproductive tract abnormalities and the decrease in sperm quality are due to increased exposure to estrogenic chemicals during development. This hypothesis is supported by an increased incidence of clinical abnormalities in the reproductive tract of human males exposed to the estrogenic drug diethylstilbestrol *in utero*, and adverse effects on testicular development and sperm quality in laboratory animals exposed to EEDs. Despite the evidence, the mechanism(s) of action of EEDs are unclear. A comprehensive strategy was used in order to assess the effects of gestational and lactational exposure to both weak (polychlorinated biphenyls; Aroclor 1242), and potent (diethylstilbestrol; DES) estrogenic chemicals on testicular development, sperm count and motility, and sperm fertilizing ability *in vitro* in both early (postnatal day (PND) 105) and middle-aged mice (PND315). In order to identify molecular pathways affected by exposure to DES, testicular

gene expression was assessed using cDNA microarrays and real-time PCR on PND21, 105 and 315. Exposure to Aroclor 1242 caused no adverse effects on testis weight or sperm count and motility, however, sperm fertilizing ability *in vitro* was significantly decreased in almost all PCB-exposed groups in both early and middle-aged mice. By contrast, exposure to DES caused a persistent decrease in the number of Sertoli cells, and modest but significant decreases in testis weight and sperm count. Sperm fertilizing ability *in vitro* was also significantly decreased on PND105 and 315. A cDNA microarray enriched for genes expressed in the mouse testis was constructed and used to identify differentially expressed genes in the testis of DES-exposed male offspring. Real-time PCR was used to verify alterations in the expression level of selected genes. The results demonstrate that adverse effects on testicular development and sperm quality were associated with transient and latent changes in testicular gene expression. Changes in the expression of genes involved in steroidogenesis, estrogen signaling, lysosomal function and testicular development were observed, suggesting multiple mechanisms by which developmental exposure to EEDs may disrupt testicular development and sperm quality. These results also demonstrate that effects on sperm fertilizing ability *in vitro* can not be predicted based on alterations in testicular development, or sperm count and motility. Early exposure to EEDs can also cause latent, and perhaps irreversible, effects on the male reproductive system, even long after the cessation of exposure.

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ABBREVIATIONS

αERKO:	Estrogen receptor alpha knockout mouse
βERKO:	Estrogen receptor beta knockout mouse
17β-HSR:	17β-hydroxysteroid reductase
3β-HSD:	3β-hydroxysteroid dehydrogenase
AF-1:	Activation function 1
AF-2:	Activation function 2
AGD:	Anogenital distance
ALH:	Amplitude of lateral head displacement
ANOVA:	Analysis of variance
APAP:	Acetominophen
AR:	Androgen receptor
ArKO:	Aromatase knockout mouse
Bad:	Bcl-associated death promoter
C/EBP:	CCAAT/enhancer binding protein
CASA:	Computer assisted sperm analysis
CCl_4:	Carbon tetrachloride
CD36:	CD36 antigen
cDNA:	Complementary deoxyribonucleic acid
CI:	Confidence limit
CREB:	cAMP response element binding protein
CSV:	Comma separated values
C_t:	Cycle threshold
Cyp11a:	P450 side chain cleavage enzyme
Cyp17:	17α-hydroxylase/C17,20-lyase
Cyp19:	Cytochrome P45019 (a.k.a. aromatase)
Dax:	DSS-AHC Region on human X chromosome, gene1
DBD:	DNA binding domain
dbEST:	Database of expressed sequence tags
DDE:	Dichlorodiphenyl dichloroethene
DDT:	1, 1, 1-trichloro-2,2-bis(p-chlorophenyl)ethane
DES:	Diethylstilbestrol
DHT:	Dihydrotestosterone
E2:	17β-estradiol
EE:	Ethynyl estradiol
EED:	Estrogen endocrine disruptor
Egr-1:	Early growth response 1
ELISA:	Enzyme-linked immunosorbent assay
ERα:	Estrogen receptor alpha
ERβ:	Estrogen receptor beta
ERE:	Estrogen response element
ERR:	Estrogen receptor-related receptor
EST:	Expressed sequence tag

FDA:	Food and drug administration
FSH:	Follicle stimulating hormone
GD:	Gestational day
GEN:	Genistein
GnRH:	Gonadotropin releasing hormone
GP3:	GenePix post-processing program
HCG:	Human chorionic gonadotropin
Hoxa10:	Homeobox A10
HPLC:	High performance liquid chromatography
HRGC:	High resolution gas chromatography
HRMS:	High resolution mass spectrometry
Hsp70:	Heat shock protein 70 kDa
Hsp90:	Heat shock protein 90 kDa
IL-2:	Interleukin 2
IL-8:	Interleukin 8
Inhbc:	Inhibin beta c
IVF:	*In vitro* fertilization
LBD:	Ligand binding domain
LGP85:	Lysosomal glycoprotein 85 kDa
LH:	Luteinizing hormone
LRMS:	Low resolution mass spectrometry
LS-mean:	Least squares mean
MSD:	Mass selective detector
NR:	Nuclear receptor
o,p-DDE:	Dichlorodiphenyl dichloroethene
OH-PCB:	Polychlorinated biphenylol
ORF:	Open reading frame
P450scc:	P450 side chain cleavage enzyme
PCB:	Polychlorinated biphenyl
PMSG:	Pregnant mare's serum gonadotropin
PND:	Postnatal day
PPM:	Part per million
Psap:	Prosaposin
PTU:	6-propyl-2-thiouracil
RA:	Retinoic acid
RNA:	Ribonucleic acid
rS14:	Ribosomal protein S14
RT-PCR:	Reverse transcriptase-polymerase chain reaction
SD:	Standard deviation
SE:	Standard error
SF-1:	Steroidogenic factor 1
SIM:	Selective ion monitoring
SOM:	Self organizing map
SOX-9:	SRY-box containing gene 9

Sparc:	Secreted acidic cysteine rich glycoprotein
SR-B1:	Scavenger receptor B1
SRC-1:	Steroid receptor coactivator 1
SREBP:	Sterol response element binding protein
Star:	Steroidogenic acute regulatory protein
T_3:	Thyroid hormone
Tr2-11:	Testicular receptor 2
UDPGT:	UDP-glucuronosyltransferase
US EPA	United States Environmental Protection Agency
Wnt-1:	Wingless-type MMTV integration site family, member 1
Wnt-7a:	Wingless-type MMTV integration site family, member 7a
Xmr:	Xlr-relates, meiosis regulated
Zpr3:	Zona pellucida protein 3

CHAPTER 1

RATIONALE, HYPOTHESIS AND AIMS

Rationale

Studies have shown that selected xenobiotics and natural products can bind to the estrogen receptor (ER) and elicit estrogenic responses in a number of *in vitro* assays and *in vivo* models. Currently, there is considerable debate regarding the degree to which human populations are being adversely affected from exposure to estrogenic endocrine disruptors (EEDs). Despite contradictory re-analyses of epidemiology studies, a mechanistically feasible hypothesis has been proposed that suggests the increased incidence of development disorders of the male reproductive tract and the decrease in sperm quality is due to increased exposure to estrogenic chemicals *in utero*. This hypothesis is supported by 1) reproductive tract abnormalities and decreased fertility observed in wildlife populations residing in areas contaminated with high levels of EEDs, 2) clinical abnormalities in the reproductive tract of human males exposed to the estrogenic drug diethylstilbestrol *in utero*, and 3) adverse effects on testicular development and sperm quality in laboratory animals exposed to EEDs. Despite the evidence, the mechanisms of action of EEDs are currently unclear. Furthermore, it is not known whether adverse effects induced by developmental exposure to EEDs persist to later stages of life. Therefore, to estimate the risk of exposure to EEDs on reproductive health in humans, a more detailed

mechanistic understanding of how developmental exposure to EEDs causes adverse effects on male reproductive health is needed.

Hypothesis:

Gestational and lactational exposure to estrogenic endocrine disruptors causes long-term alterations in testicular development and sperm quality as a result of transient and latent changes in testicular gene expression.

Aims:

1. Determine the effects of both weak (polychlorinated biphenyls: i.e. Aroclor 1242) and potent (diethylstilbestrol) EEDs on murine testicular development and sperm quality following gestational and lactational exposure.
2. Develop cDNA microarrays and analytical methods for large-scale quantitative analysis of testicular gene expression in mice.
3. Apply cDNA microarrays and real-time PCR to examine testicular gene expression in affected mice in order to establish associations between changes in gene expression and effects on testicular development and sperm quality.

CHAPTER 2

THE ROLE OF ESTROGEN IN SPERMATOGENESIS AND THE EFFECTS OF ESTROGENIC ENDOCRINE DISRUPTORS

Introduction

As early as 1974, Nelson and Bunge (1) suggested that the quality of human semen has declined over the past 50 to 60 years. In 1992, Carlsen et al. (2) published a meta-analysis of 61 studies that revealed a significant decrease in sperm concentration and seminal volume between 1938 and 1990 (from 113 x 10^6/ml in 1940 to 55 x 10^6/ml in 1990). While the results of this study were criticized by several authors for methodological reasons (3,4), subsequent studies from other laboratories have confirmed the results of Carlsen et al. (5,6). In contrast, studies published in 1996 found no decline in sperm counts within France (7) or the U.S. (8), and significant geographical differences have been detected within the U.S. (9) and between Scandinavian countries (10,11), which further confound the interpretation of the results. The incidence of testicular cancer has also increased 2 to 4 % per year during the same time period (12,13). Incidences of male reproductive tract malformations, such as cryptorchidism (undescended testes) and hypospadias (genital malformations), also appear to be slightly increasing (14).

It was recognized that these effects were similar to those observed in humans and experimental animals exposed to exogenous estrogens during fetal

life. This led Sharpe and Skakkebaek (15) to hypothesize that fetal exposure to exogenous estrogenic chemicals was compromising male reproductive health, including sperm quality and reproductive tract development. This chapter will review the mechanism of action of estrogen and the estrogen receptor (ER), spermatogenesis and testicular steroidogenesis, and the function of estrogen and the ER in spermatogenesis. Estrogenic endocrine disruptors (EEDs) will be discussed, and the effects of the EED diethylstilbestrol (DES) on testicular development and sperm quality in humans and rodent data will be reviewed.

Mechanism of action of estrogen

Endogenous estrogen (i.e. 17β-estradiol (E2)) influences the growth, differentiation, development and function of several target tissues involved in reproduction, cardiovascular performance, bone maintenance, homeostasis and behavior. Many of these processes are modulated as a result of estrogen receptor (ER)-mediated expression of estrogen responsive genes (reviewed in (16) and illustrated in Figure 1). There are two isoforms of the ER, termed ERα and ERβ, which are encoded for by two genes. The ER functions as a ligand-inducible DNA-binding transcription factor. Upon binding of ligand, the ER undergoes a conformational change to an activated form, presumably by dissociation of associated chaperone proteins such as Hsp90, Hsp70 and other proteins. The activated form of the ER dimerizes and binds to palindromic estrogen response elements (ERE) in the genome to stimulate transcription of target genes. In addition to the formation of ERα and ERβ homodimers, an ERα/β heterodimer can also form *in vitro* and *in vivo* (17). Gene transcription

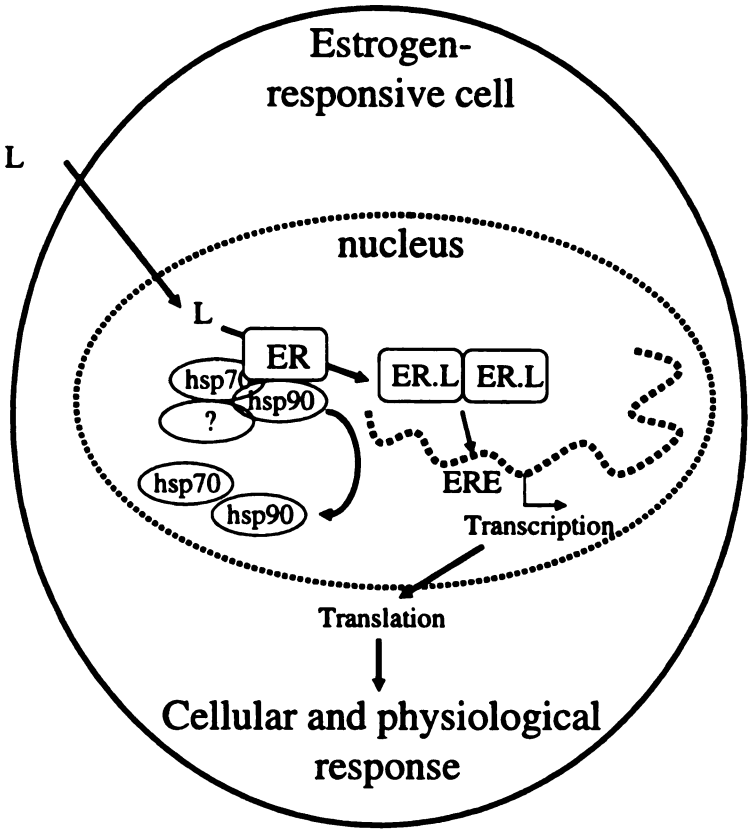


Figure 1. Estrogen receptor-mediated mechanism of action of estrogenic endocrine disruptors. ER: estrogen receptor, L: ligand, hsp: heat shock protein, ?: other associated protein(s), ERE: estrogen response element.

stimulated by the ERE-bound, liganded-ER is believed to occur via interaction with coactivator proteins and subsequent recruitment of basal components of the transcription initiation complex and RNA polymerase II (18). In addition to ERE-mediated effects on gene expression, the ER can also activate gene transcription through an AP-1 site and the corresponding Fos/Jun complex bound to the AP-1 site (19). The transactivation activity of ERα and ERβ can also be influenced by this interaction. For example, E2 activates transcription via ERα and an AP-1 site, whereas E2 inhibits transcription via ERβ and an AP-1 site (20). In addition to ligand-induced effects on transcription, the ER can also be activated in a ligand-independent manner via phosphorylation by growth factor-induced signaling cascades (21). Effects on other proteins, such as the heat shock protein complex, corepressors and/or coactivators, may also play a role in ligand-independent ER activation.

Like other nuclear receptors, the ER is composed of six modular domains, designated A to F from the amino terminal start site to the carboxy terminal end of the protein (16). A schematic representative of the structural and functional domains of ERα and ERβ is shown in Figure 2. The highly variable A/B region contains the ligand-independent activation function (AF-1) that activates transcription constitutively and in a cell- and gene-specific manner via interaction with coactivators. The highly conserved C domain contains two zinc-fingers that recognize the ERE, and also function in dimerization and nuclear translocation. The D domain is a flexible hinge region that separates the DNA binding domain

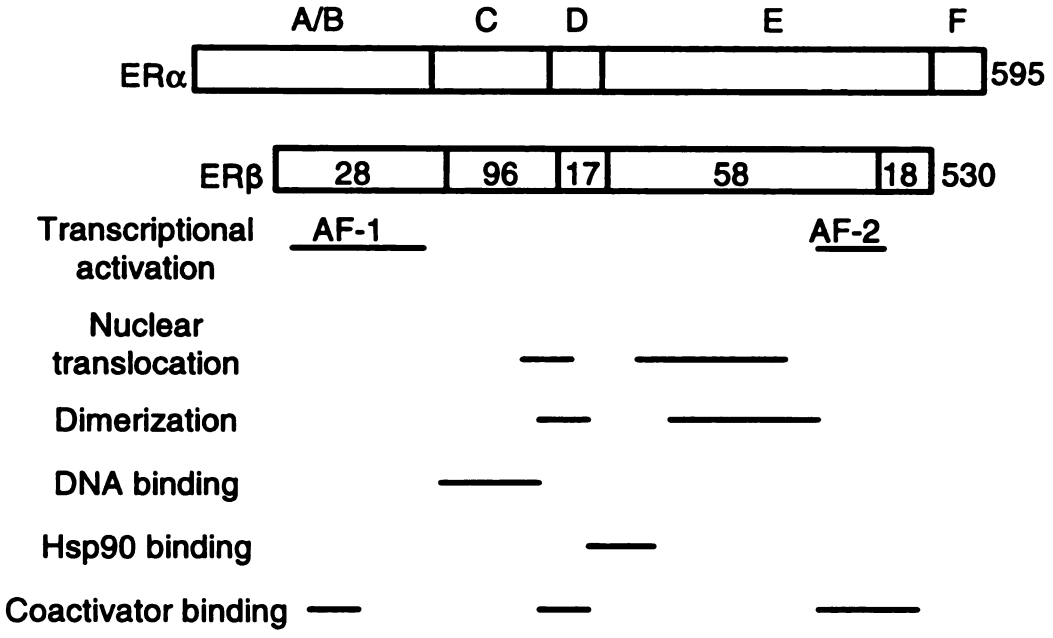


Figure 2. Schematic representation of the modular structure of the human estrogen receptor alpha and beta. The domains are labeled A through F for the human ERα and ERβ. The numbers on the right indicate the length of the peptide in amino acids. The numbers within the domains indicate the percent amino acid identity between ERα and ERβ. Adapted from (16).

(DBD) from the ligand binding domain (LBD). The LBD consists of domains E and F, and functions in nuclear translocation, hsp90 binding, and dimerization. The LBD also contains the ligand-dependent activation function (AF-2) that modulates transcription via interaction with coactivators or corepressors (22). The function of the F domain is unclear, but has been shown to influence ligand binding and interaction with coactivators (23).

Spermatogenesis and testicular steroidogenesis

The testis is a complex organ composed of three major cell types: Leydig cells, Sertoli cells and germ cells. The Sertoli cells and germ cells are contained within the seminiferous tubules, which separate sperm from the interstitial compartment that contains the venous and lymphatic system, in addition to the Leydig cells (Figure 3). Sertoli cells are highly specialized cell types that extend from the basal lamina to the luminal surface of the seminiferous tubules. In addition to providing a structural scaffold to sequester developing sperm, the Sertoli cells secrete a number of proteins and nutrients that regulate the initiation and progression of spermatogenesis. Sertoli cells also form tight junctional complexes that provide a blood-testis barrier to protect germ cells from immunological attack. The principal function of the Leydig cell is to produce testosterone from cholesterol precursors (Figure 4). Steroidogenesis begins with the import of cholesterol into the cell via SR-B1 (for esterified cholesterol) or CD36 (for free cholesterol). Cholesterol can also be synthesized *de novo* via the sterol response element binding protein (SREBP).

Figure 3. Schematic representation of a cross-sectional view of the testis and the seminiferous tubules. Only spermatozoa are shown within the seminiferous tubules. Venous system is not shown, but is within the interstitial space. The Sertoli cells create basal and adluminal compartments by virtue of tight juctional complexes, which separate the immune system from the germ cells undergoing meiosis in the adluminal compartment.

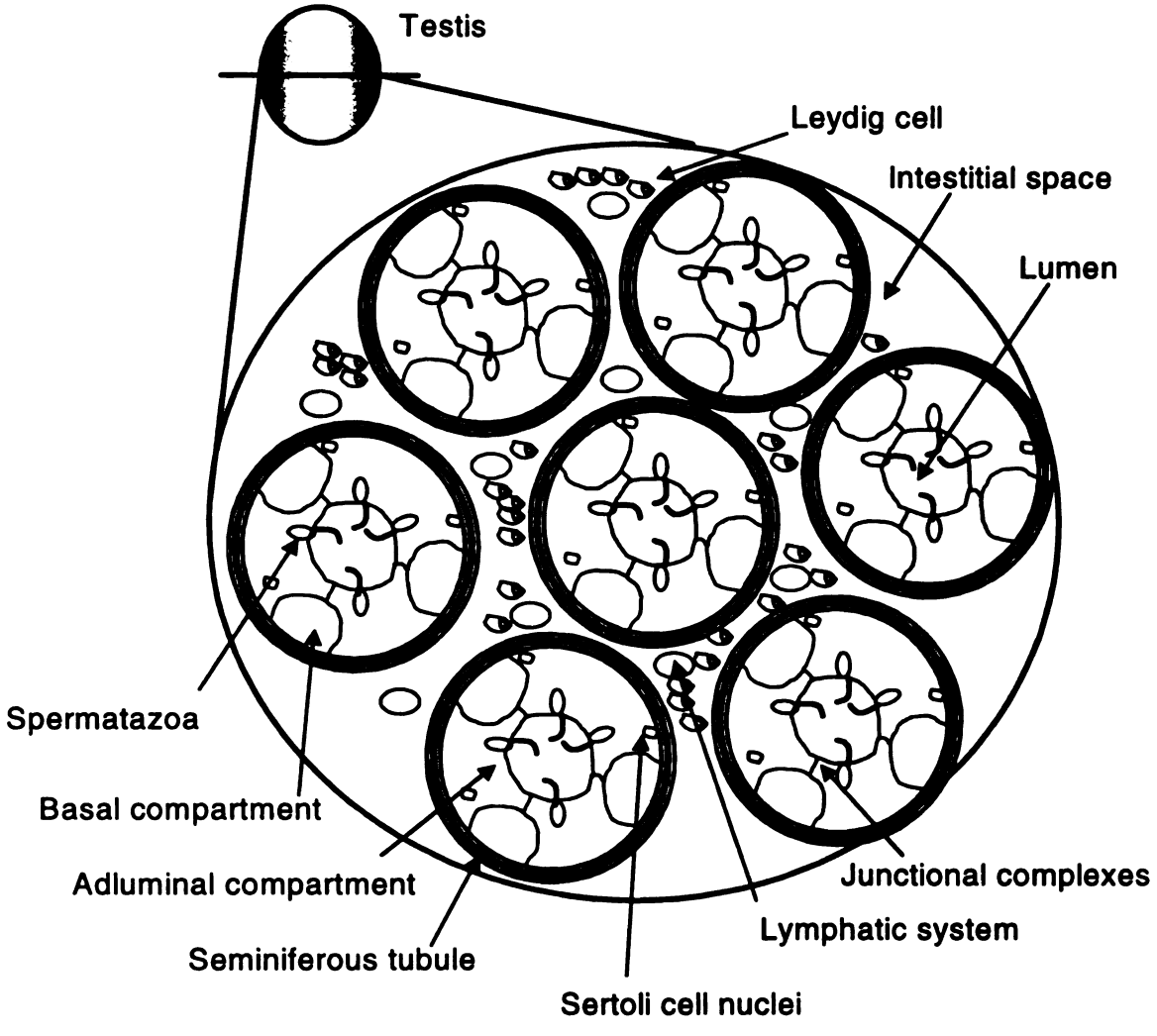
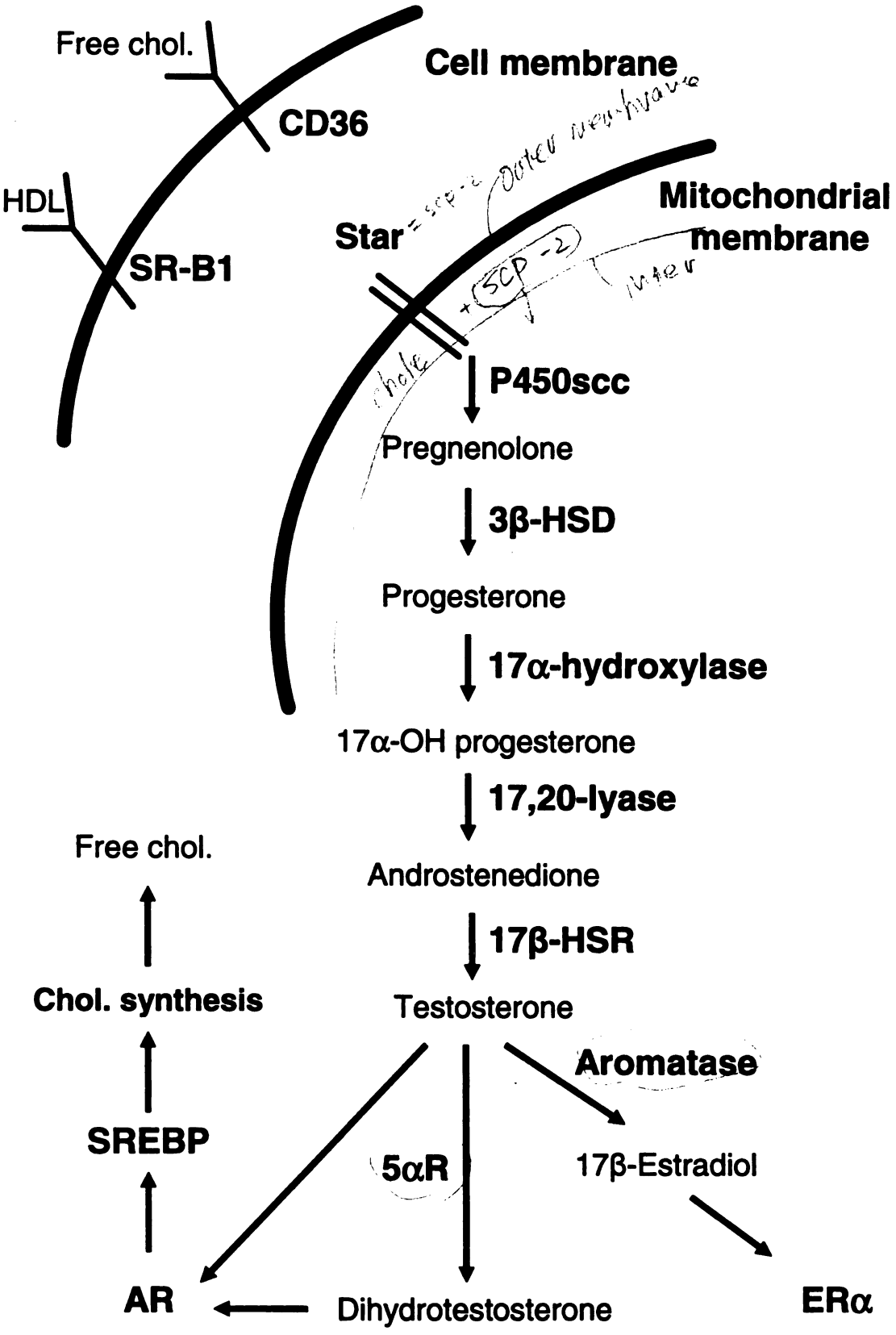

Testis
Leydig cell
Intestitial space
Lumen
Spermatazoa
Basal compartment
Adluminal compartment
Seminiferous tubule
Sertoli cell nuclei
Lymphatic system
Junctional complexes

Figure 4. Steroidogenic pathway in the Leydig cells of the testis. De novo synthesized cholesterol, or free (Free chol.) and esterified cholesterol (HDL) from outside the cell, is transported across the mitochondrial membrane by Star (Steroidogenic acute regulatory protein) where it is converted to pregnenolone by P450scc (Cytochrome P450 side chain cleavage enzyme), the rate limiting step in the steroidogenic pathway. A series of enzymatic reactions convert pregnenolone to testosterone. SR-B1: scavenger receptor B1, CD36: CD36 antigen, 3β-HSD: 3β-hydroxysteroid dehydrogenase, 17β-HSR: 17β-hydroxysteroid reductase, ERα: estrogen receptor α, AR: androgen receptor, SREBP: sterol response element binding protein, 5αR: 5α-reductase. Note that 17,20-lyase is the same enzyme as the 17α-hydroxylase.


Free chol.
CD36
Cell membrane
HDL
SR-B1
Star
Mitochondrial membrane
P450scc
Pregnenolone
3β-HSD
Progesterone
17α-hydroxylase
17α-OH progesterone
17,20-lyase
Androstenedione
17β-HSR
Testosterone
Free chol.
Chol. synthesis
SREBP
AR
5αR
Aromatase
17β-Estradiol
ERα
Dihydrotestosterone

Cholesterol is transported through the mitochondrial membrane via the steroidogenic acute regulatory protein (Star). Cholesterol is then cleaved by the P450 side chain cleavage enzyme (P450scc) to form pregnenolone, where it is sequentially converted into testosterone. Smaller amounts of dihydrotestosterone (DHT) and E2 are also produced by testicular and extragonadal conversion of testosterone by 5α-reductase and aromatase enzymes, respectively.

Spermatogenesis is the process whereby diploid germ cells develop into haploid spermatozoa, which subsequently travel to the epididymis where they gain motility and the ability to fertilize an egg. Primordial germ cells, or spermatogonia, are the stem cell population that can divide many times by mitosis (spermatocytogenesis), thereby maintaining a large reserve pool of germ cells that can ultimately commit to meiosis and become haploid spermatozoa. Immature spermatogonia precursors (type A_d) progressively differentiate into more mature spermatogonia (type A_p and B). After entering meiosis, each preleptotene spermatocyte undergoes two reductive divisions to form four haploid round spermatids (spermatogenesis). The final process involves differentiation of round spermatids to elongated spermatozoa (spermiogenesis), which are characterized by compacted DNA, a flagellum for motility, and an acrosome for oocyte penetration. The whole process of gametogenesis, from the spermatogonia stage to the spermatozoa stage, occurs from the basal to the adluminal compartment of the seminiferous tubules. The spermatozoa are

released into the lumen of the seminiferous tubule and travel to the epididymis, where they undergo further maturation and gain the ability to fertilize an egg.

Role of estrogen in spermatogenesis

Estrogen has long been viewed as a female sex steroid, although it has been known for some time that E2 binding sites have been detected in the fetal, neonatal and adult reproductive tract of the male (24,25). Prenatal or neonatal exposure to estrogen impairs normal development and function of the male reproductive tract (26). Low doses of estrogen to adult male rats can also lower serum luteinizing hormone (LH) and testosterone levels, and decrease accessory tissue weights (i.e. epididymis, seminal vesicle) and testicular sperm count (27). These studies indicated estrogen plays a role in regulating development and function of the male reproductive tract, however, it was not until recently that the role of ERα and ERβ in spermatogenesis was confirmed.

With the cloning of both isoforms of the ER (28,29), and the generation of antibodies, the ontogeny and cell-specific expression pattern of both ER isoforms have been established within the testis and other tissues of the male reproductive tract in a variety of species (30). In the mouse, the expression of ERα mRNA and protein appears to be confined to the Leydig cells throughout early development and adulthood (30). The expression of mouse ERβ mRNA has been detected in the fetal and neonatal testis (30), but not in the adult testis (31). By contrast, immunoreactivity of ERβ has been detected in Leydig cells and elongated spermatids of adult mice (32), as well as spermatocytes of the developing testis (30). Furthermore, ERβ has been detected in Sertoli cells and

spermatagonia of neonatal and adult rats (33). Additional differences in the ontogeny and cell-specific expression pattern of ERα and ERβ have been reported between mouse and rat, and other species (34-36). These differences in mRNA and protein localization may be due to differences in species, strain, antibodies or methods of detection. The discrete expression pattern of the ER in the testis of all mammalian species examined supports a crucial role of the ER in spermatogenesis or reproductive tract development. Conclusive evidence, however, would not come until the generation of ER knockout mice.

The reproductive tract of male mice homozygous for a mutation in the ERα gene (αERKO mice) appears anatomically normal, however, the mice are infertile (37). The testes of adult αERKO mice are smaller and the seminiferous tubules are degenerate and atrophic. This disruption does not appear until approximately 20 days of age, and appears to further deteriorate with age. Nonetheless, spermatogenesis progresses normally in prepuburtal and young adult αERKO mice, and similar numbers of cauda epididymal sperm are present until 10 weeks of age. Despite the appearance of normal spermatogenesis, epididymal sperm from 8 to 16 week old αERKO mice are less motile and ineffective at fertilizing eggs *in vitro*. Subsequent studies established that disruption of ERα leads to dilation of the rete testis and efferent ductules in the head of the epididymis, which results in accumulation of fluid in the seminiferous tubules, dilution of sperm, and infertility (38). Thus, ERα regulates genes involved in reabsorption of fluid for concentrating sperm in the efferent ducts and head of the epididymis. These observations led to the discovery that ERα

regulates expression of the sodium/hydrogen exchange-3 protein, which is necessary for fluid reabsorption in the efferent ducts (39). To determine whether ERα is required by somatic or germ cells of the male reproductive tract, germ cells from αERKO mice were transplanted into wild-type mice depleted of germ cells by busulfan treatment (40). Recipient mice sired heterozygous offspring, which when mated, produced αERKO mice with the same phenotype as originally reported for αERKO mice. These results indicate that ERα is required by the somatic cells of the reproductive tract in order to support the production of sperm that are capable of fertilization. By contrast to αERKO mice, mice homozygous for a mutation in the ERβ gene (βERKO) exhibit no apparent reproductive tract or behavioral abnormalities and are as fertile as wild-type mice (41). Despite the expression of ERβ in the testis, the functional importance of ERβ is unknown.

Although ERα expression in the somatic cells of the male reproductive tract is required for fertility, the role of E2 in spermatogenesis and fertility was until recently unclear. The aromatase enzyme, which converts testosterone to E2, is known to be expressed within the somatic and germ cells of the testis (42), thus raising speculation that local E2 synthesis is required for spermatogenesis. Male mice homozygous null for the aromatase gene (ArKO) were originally reported to be fertile with histologically normal testes at 9 weeks of age (43). However, subsequent examination of older ArKO mice revealed that they develop progressive infertility between 4.5 months and 1 year (44). Spermatogenesis in ArKO mice is arrested at early spermatogenic stages, with

increased apoptosis and a loss of round and elongated spermatids. Early germ cells and Sertoli cells were normal, and Leydig cells were hypertrophic, likely as a result of increased LH levels. The infertility of ArKO mice is believed to be due to the impairment of round spermatids to differentiate into mature spermatazoa. Collectively, gene knockout experiments have demonstrated that local estrogen synthesis and action within the male reproductive tract is crucial for the development of spermatozoa that are capable of fertilizing oocytes.

Estrogen endocrine disruptors

Laboratory experiments have demonstrated adverse effects on reproductive tract development and fertility in animals exposed to exogenous estrogenic chemicals during perinatal (*in utero* and neonatal) development (45). In addition to exogenous estrogens, other chemicals that mimic or inhibit the action of androgen have also been observed to disrupt the male reproductive system in laboratory animals (46,47). These chemicals have collectively been termed endocrine disruptors. An endocrine disruptor can be defined as an exogenous agent that elicits adverse health effects in an intact organism or its progeny, secondary to changes in endocrine function (48). A considerable amount of attention has been given to the large number of exogenous chemicals that can mimic or inhibit the action of estrogens. These chemicals are commonly referred to as estrogenic endocrine disruptors (EEDs), and encompass a diverse group of compounds including naturally occurring products (mycotoxins, isoflavones), environmental pollutants (polychlorinated biphenyls, polyaromatic hydrocarbons), pharmaceuticals (synthetic estrogens and antiestrogens),

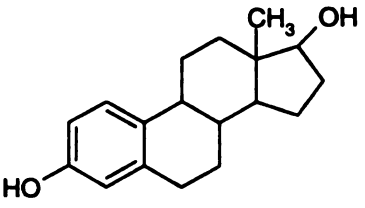
pesticides (o,p-DDT, dieldrin) and industrial chemicals (bisphenol A, octylphenol) (Figure 5).

Changes in endocrine function may result from chemicals that interfere with receptor binding, secretion, synthesis, transport, or elimination of hormones necessary for normal function and homeostasis of reproduction, development and/or behavior. Possible human health effects include breast cancer and endometriosis in women, prostate and testicular cancer in men, as well as abnormal sexual development, reduced fertility, immune suppression and neurobehavioral effects. The greatest concern over EEDs is that exposure during critical periods of development may lead to adverse effects on reproductive health at later stages of development. The allegation that EEDs can adversely affect human reproductive health is contentious due to the relatively weak potency and low level of exposure to EEDs in comparison to dietary derived phytoestrogens, and the relatively high dose of EEDs necessary to cause adverse effects in laboratory animals (49-51).

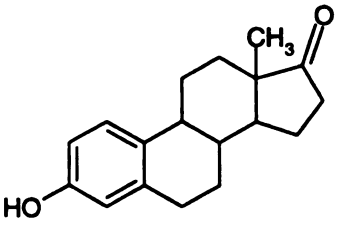
In response to the concern over EEDs, the Safe Drinking Water Act Amendment and the Food Quality Protection Act were introduced, which require the United States Environmental Protection Agency (US EPA) to test all chemicals for estrogenic, androgenic and thyroid-like activity. It was the opinion of the US EPA's Science Policy Council that, with a few exceptions (i.e. DES), a causal relationship between exposure to specific EEDs and adverse health effects in humans has not been established (51). It was recommended,

Figure 5. Structures of endogenous and exogenous estrogens with endocrine disrupting activity. Examples include A) endogenous estrogens, B) naturally occurring products, C) environmental pollutants, D) pharmaceuticals, E), pesticides (o,p-DDT, dieldrin) and F) industrial chemicals.

A Endogenous estrogens

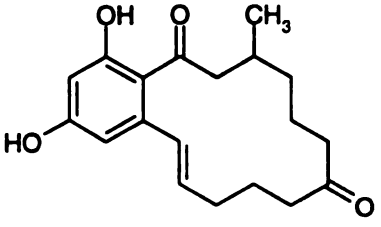


17β-estradiol

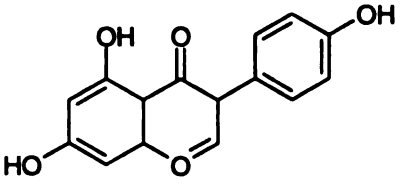


Estrone

B Naturally occurring products

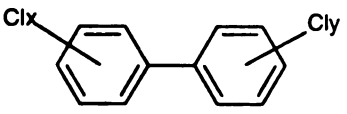


Zealalenone (mycotoxin)



Genistein (isoflavone)

C Environmental Pollutants



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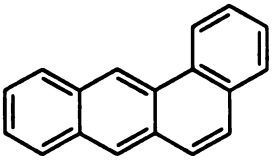
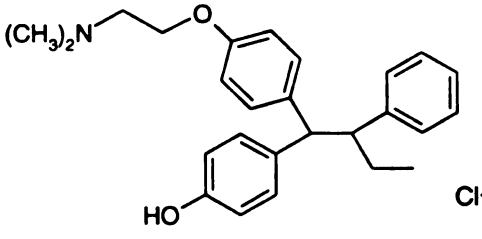
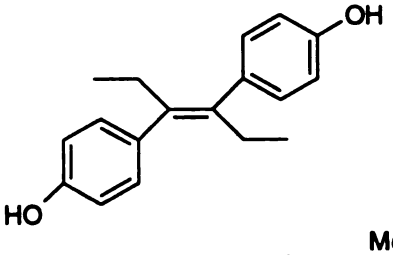


Benzo[a]pyrene

D Pharmaceuticals

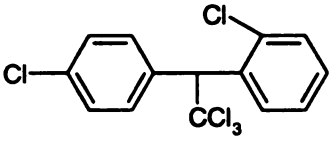


Tamoxifen

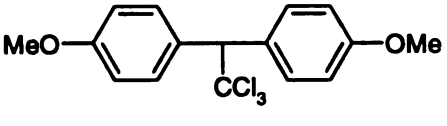


Diethylstilbestrol

E Pesticides

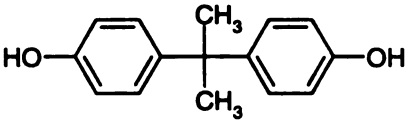


o,p-DDT

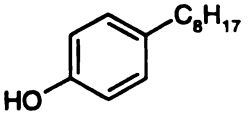


Methoxychlor

F Industrial Chemicals



Bisphenol A



Octylphenol

however, that new epidemiologic and laboratory studies be undertaken in order to better define the scope of the problem, to develop and validate short-term screening studies in an effort to elucidate the mechanisms of action of EEDs, and to develop predictive biomarkers indicative of an adverse effect (51,52).

Diethylstilbestrol and its effects on human males exposed *in utero*

Diethylstilbestrol (DES) is a non-steroidal synthetic estrogen that was administered to pregnant women to prevent miscarriages in the late 1940's and 1950's. It has been estimated that at least 2 million people between 30 and 45 years of age have been exposed to DES *in utero* (53). It has also been used to suppress lactation, control menopausal symptoms, treat breast and prostate cancer, and as an abortificient (54). Its use was terminated in 1971 when it was concluded that prenatal exposure to DES causes vaginal and cervical clear-cell adenocarcinoma in female offspring following otherwise normal pubertal development (55,56). Since then, females exposed to DES *in utero* were found to have an increased incidence of infertility, miscarriage, preterm delivery and fetal or infant death (53,57,58). Although an increase in the incidence of reproductive tract abnormalities have been noted, the effect of prenatal DES exposure on male fertility is equivocal.

Table 1 summarizes the clinical observations of human males exposed *in utero* to DES. The dose (1.4 to 17.9 g total dose during pregnancy) and timing of DES administration to pregnant women were highly variable. Thus, it is difficult to establish the minimum dose or critical period of exposure necessary to induce adverse health effects in male or female offspring. There was widespread use of

Table 1. Summary of the non-malignant clinical observations in human males exposed to diethylstilbestrol *in utero*.

Study	Clinical Finding	DES – (Number of subjects)	DES+ (Number of subjects)
Dieckmann cohort (65)	Epididymal cysts, hypotrophic testes, and capsular induration	6.1 % (168)	25.1 % (163)
	Ejaculate volume < 1.5 ml	0 % (25)	26 % (39)
	Severely pathological semen	0 % (25)	28 % (39)
SF Bay area cohort (66)	Urogenital abnormalities	8 % (24)	13 % (24)
	Severe pathological change in sperm (Eliasson score > 10)	20 % (24)	17 % (24)
	Sperm density (< 10^6/ml)	17 % (24)	9 % (24)
Dieckmann cohort (67)	Cryptorchidism	0.3 % (307)	5.5 % (308)
	Urogenital abnormalities	7.8 % (51)	31.5 % (51)
	Severe pathological change in sperm (Eliasson score > 10)	8 % (51)	18 % (51)
Seattle cohort (68)	Cryptorchidism	0 % (29)	8 (51)
	Urogenital abnormalities	4 % (51)	35 % (51)
	Severe pathological change in sperm (Eliasson score > 10)	0 % (51)	21 % (51)
Dieckmann cohort (61)	Urogenital abnormalities	5 % (241)	27 % (253)
LA cohort (69,70)	Cryptorchidism	0.9 % (111)	1.3 % (225)
	Difficulty passing urine Hypospadias or penile stenosis	1.8 % (111)	12.9 % (225)
Rochester cohort (71)	Hypospadias	1.1 % (274)	0 % (265)
	Testes anomalies[a]	1.1 % (274)	3.0 % (265)
	Epididymal cysts	5.1 % (274)	6.9 % (262)
	Capsular induration	0 % (274)	0 % (262)
	Semen analysis[b]	- (95)	- (110)

[a] Includes agenesis, atrophy, cryptorchidism persisting for one year.
[b] No significant differences between control and DES-exposed.

the dosing regime recommended by Smith et al. (59). As a result, most human data are related to the high dose regimens. The results of clinical findings demonstrate a strong correlation between DES exposure and urogenital abnormalities (Table 1). The effects on sperm quality are less conclusive, however, and effects on fertility have been suspected but not thoroughly studied. Using a sperm penetration assay, 14 of 17 DES-exposed men and 2 of 12 non-exposed men had scores of less than 14 % and therefore qualified as infertile (60). The most recent fertility study on DES-exposed men found no impairment of fertility by any measure, or any reported decline in sexual behaviors (61). However, measures of human male fertility, such as age at the birth of their first child, average number of children, medical diagnosis of a fertility problem, length of time to conception, or whether they had ever impregnated a woman, are indirect measures of sperm function and quality. Other behavioral, social, and environmental factors could influence these measures.

In addition to infertility, an association between DES exposure and testicular cancer has also been suspected (62-64). Case-control studies, however, have been inconsistent. Recently, a large study of 3613 DES-exposed and unexposed individuals found no increased relative rate for overall cancers between DES-exposed and unexposed men, or even versus national cancer rates (72). However, the relative rate of testicular cancer in DES-exposed men was 3.05 (95% CI = 0.65 to 22.0) times greater than unexposed men (95 % CI = 0.82 to 4.20) and the general population rate. The authors of this study conclude that these results are compatible with chance occurrence, and it is unlikely that

DES exposure plays a causative role in the increased rate of testicular cancer observed in developing countries over the past 60 years (13). However, DES-exposed men are not yet 50 years old, and adverse effects on fertility or cancer may manifest themselves at later stages of life. Further monitoring of this exposed population will be required in order to confirm or refute the equivocal clinical findings.

The effect of *in utero* exposure to diethylstilbestrol on testicular development and sperm quality in laboratory animals

Because of the high potency of DES as an estrogen agonist, and the availability of human clinical data from DES-exposed males, DES has become a prototypical EED and has been widely used for characterizing the effects of exposure to estrogens on male reproductive tract development (73). In 1975, McLachlan (74) reported that prenatal exposure of male mice to high doses of DES (100 µg/kg maternal body weight) during gestation resulted in 60 % of the mice being sterile. This was likely due to the fact that 75 % of the mice had reproductive abnormalities, including cryptorchidism, hypoplastic and fibrotic testes, and epididymal cysts. Reproductive abnormalities, however, were not observed in lower dose groups (10, 1, 0.01 µg/kg). Subsequent studies have demonstrated that prenatal exposure of male mice to DES (≥100 µg/kg maternal body weight) causes sterility and a number of reproductive tract abnormalities, including enlarged and cystic Müllerian remnants, inhibition of gubernaculum development and cryptorchidism, sperm granulomas, hypotrophic testes and epididymides, epididymal cysts of embryonic female origin, and tumors of the

rete testis and interstitial cells (75-80). These lesions likely contribute directly to sterility since adverse effects on fertility at lower doses (<100 µg/kg) or doses that do not cause reproductive tract abnormalities have not been described. For example, gestational and lactational exposure of rats to 50 µg/l DES in drinking water (~8.6 µg/kg/day) caused a small but significant decrease in testis and epididymis weight and testicular and epididymal sperm counts (81). Sharpe et al. have also observed small but significant decreases in testes weight and daily sperm production in rats exposed through gestation and lactation to 100 µg/l DES in drinking water (82). However, these results were not confirmed in repeat studies (83). In any event, it is unclear whether gestational and lactational exposure to DES, or other EEDs, can adversely effect male fertility at non-teratogenic doses since changes in sperm production or organ weights do not adequately predict sperm quality (84,85).

The size of the testis, the number of Sertoli cells, and germ cell production in adulthood are highly correlated with the number of Sertoli cells produced during the perinatal period, which is positively controlled by FSH (86-88). FSH secretion is inhibited by E2 in the pituitary via aromatization of testosterone produced in the testis. Therefore, Sharpe and Skakkebaek (15) have hypothesized that *in utero* exposure to exogenous estrogens can decrease adult sperm counts as a result of feedback inhibition of gonadotropin secretion during the perinatal growth phase (Figure 6). This hypothesis is supported by studies demonstrating that neonatal treatment of rats with DES or gonadotropin releasing hormone (GnRH) antagonists can cause a decrease in Sertoli cell number and

sperm production in adulthood (89-91). There is also evidence that perinatal exposure to DES can inhibit testosterone production and/or action. For example, *in utero* exposure of mice to DES reduces testicular expression of 17α-hydroxylase/C17,20 lyase, a key enzyme in testosterone production (92). Neonatal and post-pubertal exposure to DES has also been shown to induce long-term decreases in testosterone production (91,93,94). However, the unique morphological abnormalities induced by DES, in contrast to GnRH antagonists (91), suggests that DES is acting through other mechanisms, in addition to the inhibition of gonadotropin secretion (Figure 6). Due to the role of the ER as a transcription factor, it is expected that DES may cause alterations in testicular gene expression. Therefore, determining estrogen responsive genes in the testis may lead to an enriched molecular understanding of how estrogen affects spermatogenesis, and how exposure to DES, and potentially other EEDs, may disrupt testicular development and compromise sperm quality.

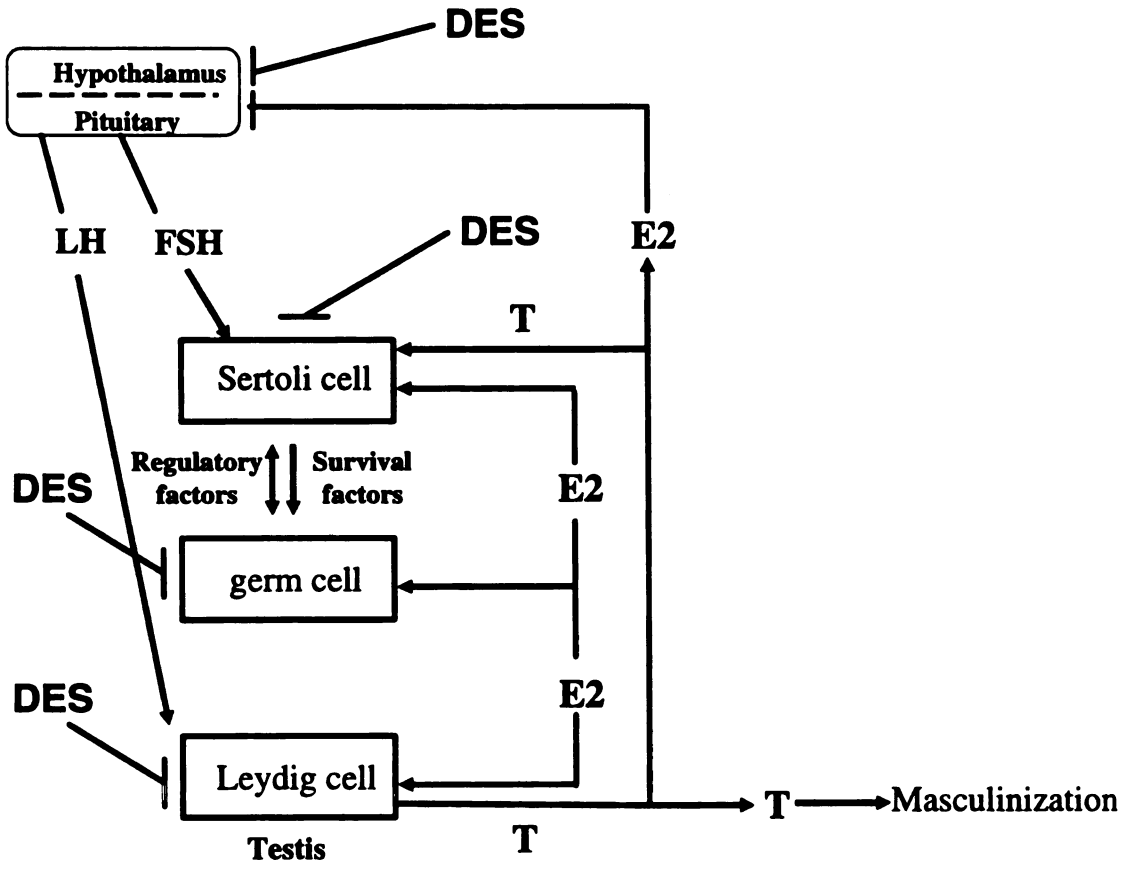


Figure 6. Hormonal regulation of spermatogenesis in the testis. Follicle stimulating hormone (FSH) secreted by the pituitary stimulates proliferation of Sertoli cells during the perinatal growth phase. The number of Sertoli cells produced ultimately dictates the number of germ cells the testis can support, and sperm count in adulthood. Luteinizing hormone (LH), also secreted from the pituitary, stimulates steroidogenesis in the Leydig cells. Testosterone (T) regulates Masculinization of the Wolffian ducts and the external genitalia. T can also be aromatized to 17β-estradiol (E2) and inhibit gonadotropin (FSH/LH) secretion in the pituitary in a negative feedback loop. Locally synthesized E2 is also thought to regulate spermatogenesis in a paracrine and/or intracrine manner. Diethylstilbestrol (DES) could potentially alter cellular function in the pituitary, Sertoli cell, Leydig cell or germ cell.

CHAPTER 3

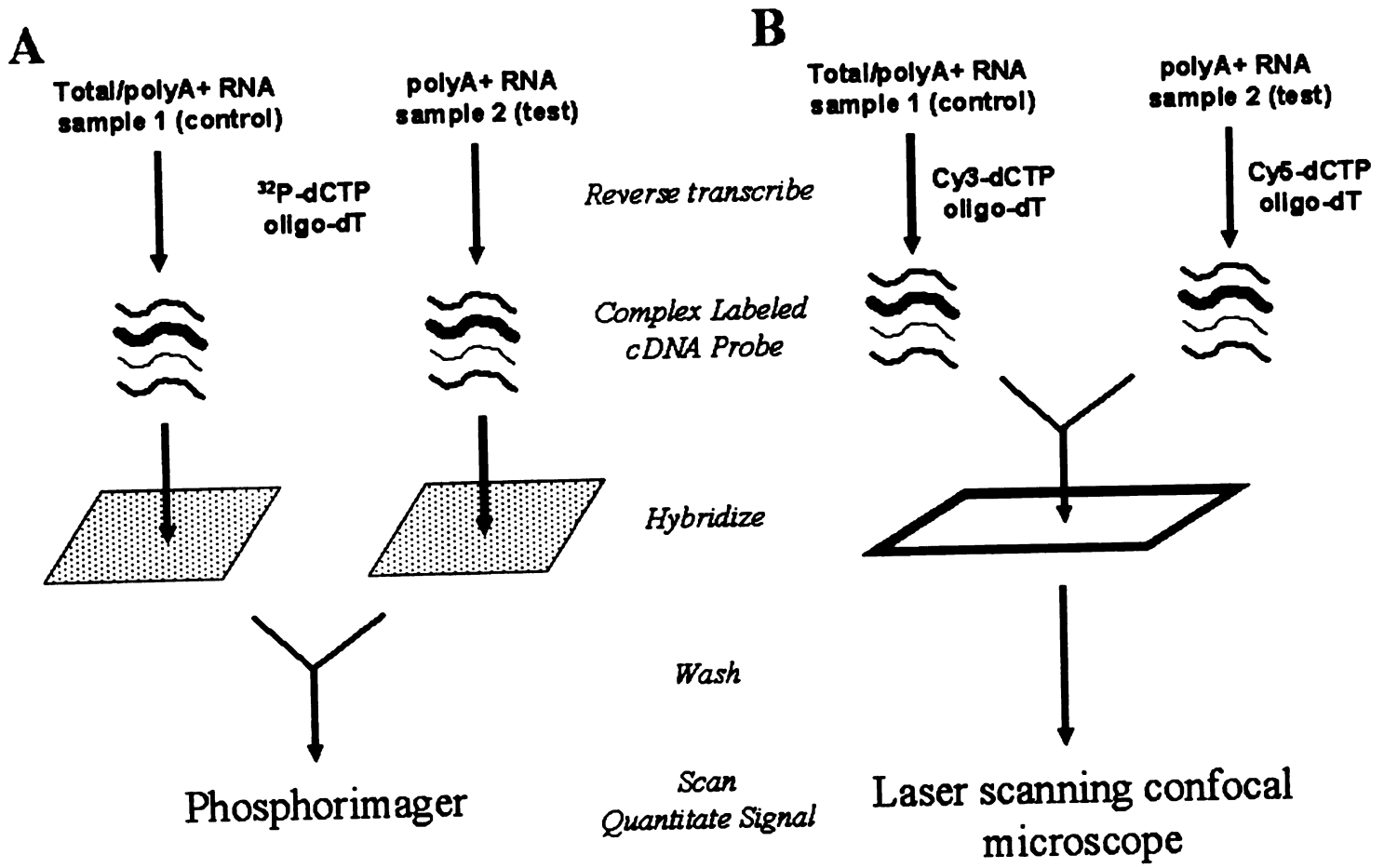
THE USE AND CHALLENGES OF DNA MICROARRAYS IN PREDICTIVE AND MECHANISTIC TOXICOLOGY[1]

Introduction

DNA microarrays have quickly emerged as the premier tool for enabling genome-wide analysis of mRNA expression (Figure 1). With microarrays, the level of mRNA expression for hundreds to tens of thousands of transcripts can be measured simultaneously in a single experiment. By contrast, Northern blot and reverse transcriptase – polymerase chain reaction (RT-PCR) methods allow for the quantitation of only a few genes at most in any one experiment. With knowledge of gene expression in cells, tissues, organs or whole organisms under a variety of physiological and pathological states, new understanding of the molecular basis of physiology, disease and toxicity can be acquired. As a result, these new technologies are influencing drug discovery and preclinical safety in the biotechnology and pharmaceutical industry. Toxicologists are also promoting genomic expression technologies as a superior alternative to traditional rodent bioassays to identify and assess the safety of chemicals, including potential EEDs and drug candidates (95,96). Ultimately, toxicogenomics (the interdisciplinary field of genomics, bioinformatics and toxicology) is expected to

[1] Portions of this chapter have been published in Fielden M.R. and Zacharewski T.R. (2001) Challenges and limitations of gene expression profiling in mechanistic and predictive toxicology. Toxicological Sciences. 60: 6-10.

Figure 1. Overview of microarray methodologies for highly parallel quantitation of gene expression. In this technique, an entire total or polyA+ RNA population from a tissue source of interest is reverse transcribed with an oligo(dT) primer in the presence of A) radiolabeled nucleotides to generate a complex labeled cDNA probe, such that the abundance of individual mRNA transcripts is reflected in the cDNA product. The labeled probes are then hybridized to an excess amount of double-stranded denatured target cDNA arrayed on a solid support, such as a nylon membrane. The arrayed cDNA contains previously cloned and partially sequenced genes and expressed sequences from a cDNA library. The microarrays are washed to remove unbound probe before being scanned on a phosphorimager to detect and quantitate radioisotopic signal intensity. The intensity of the hybridization signal is proportional to the abundance of cDNA derived from the mRNA population. Two microarrays (e.g. control and test) can then be compared to determine the presence and relative abundance of hundreds to thousands of mRNA transcripts in a single hybridization experiment. B) An alternative method, made popular by Patrick Brown and colleagues, is based on competitive hybridization of fluor-labeled probes to cDNA arrayed at high density on glass slides (97,98). In this method, control and test mRNA samples are reverse transcribed in the presence of an oligo(dT) primer and one of two modified nucleotides that fluoresce at a characteristic wavelength (e.g. Cy3-dCTP or Cy5-dCTP). The control and test fluor-labeled cDNA probes are mixed and compete for hybridization to their complementary sequence arrayed on the glass slide. After unbound probe is washed off, the microarray is scanned with a laser scanning confocal microscope. The relative fluorescent signal at each wavelength is proportional to the mRNA abundance in each sample. The relative abundance of mRNA is represented by a ratio of the fluorescent intensity at each wavelength, which indicates the fold change in gene expression in the test sample relative to the control.


A
Total/polyA+ RNA
sample 1 (control)
polyA+ RNA
sample 2 (test)
32P-dCTP
oligo-dT
Reverse transcribe
Complex Labeled
cDNA Probe
Hybridize
Wash
Scan
Quantitate Signal
Phosphorimager
B
Total/polyA+ RNA
sample 1 (control)
polyA+ RNA
sample 2 (test)
Cy3-dCTP
oligo-dT
Cy5-dCTP
oligo-dT
Laser scanning confocal
microscope

accelerate drug development and aid risk assessment for drugs, agrochemicals, and industrial chemicals. It has been proposed that each chemical that acts through a particular mechanism of action will induce a unique and characteristic gene expression profile under a given set of conditions (96). Microarray experiments applied to tumor samples, for example, have demonstrated the potential of gene expression profiling to accurately classify disease phenotypes based on gene expression alone (99-101). Therefore, it is expected that gene expression profiling can be used to classify chemicals by the similarity of their expression profiles compared to expression profiles obtained from known chemicals with defined mechanisms of action. Although many cell-based assays allow for the rapid identification of chemicals that can act through a known target or pathway, large-scale comparison of gene expression profiles has the potential to determine mechanisms of action of uncharacterized chemicals without prior knowledge of potential targets or toxicities. Due to the various mechanisms by which chemicals can disrupt the endocrine system, screening for a mechanism of action, based on expression profiles, rather than screening against a single activity provides a more comprehensive assessment of the potential for EEDs to disrupt the endocrine system.

Pairwise-conditional expression analysis

Since changes in gene expression following chemical exposure can precede and/or follow toxicity, gene expression profiling using microarrays has been recognized as a valuable tool to monitor the totality of effects on gene expression and thus potentially explain the molecular basis of toxicity (96,102).

In the simplest experimental case, the RNA population of a control sample is compared to a test sample. In these instances, the interest is in determining what genes are significantly changed and how they are changed following treatment.

In evaluating the significance of differential gene expression in microarray experiments comparing two RNA populations, an *ad hoc* threshold is usually applied to indicate the level of differential expression needed to be deemed significant. This threshold level is often chosen on the basis of observed variability in control versus control hybridization experiments. For example, a gene that does not differ in expression between two samples will have a theoretical expression ratio of one. In practice however, the observed expression ratios for > 95 % of the genes in a control versus control experiment typically range from 0.5- to 2-fold, due to experimental error. As a result, threshold values ranging from ± 1.5- to 3-fold are typically applied, depending on the variability in the system. This would result in many genes below the threshold level being disregarded and more confidence given to genes with the highest level of differential expression. This empirical method of determining significant changes in gene expression is in contrast with statistical methods of estimating significance levels (i.e. p values) for differential expression where genes are ranked in order of confidence. In order to estimate p values, replicate experimental data is required to estimate experimental error on a gene by gene basis, since some genes exhibit more variability than others. In single experiments without replication, it is common practice to assume that a subset of

genes do not change in expression, regardless of treatment. The variability in measurement of these control or "housekeeping" genes can then be used as a basis for estimating experimental error for the rest of the population of genes, thus allowing for significance levels to be calculated for the remaining genes (103). Selection of housekeeping genes are often based on historical or empirical evidence (98), however, many so called housekeeping genes have been observed to violate the assumption of constancy (104,105). Nonetheless, this method is advantageous when replication is not possible for logistical or technical reasons, such as with tumor biopsies. In order to overcome any assumptions regarding the distribution of ratio measurements and constancy of housekeeping gene expression, estimating experimental error is best achieved empirically by measuring the variation in gene expression, on a gene by gene basis, across replicate control samples (106). In many cases, results of microarray experiments are not replicated, or are measured only in duplicate. It is important to realize that pooling of samples reduces the number of replicates to one and precludes estimation of experimental error. Pooling samples is advantageous and sometimes necessary when RNA is limited due to small tissue size, although advances in cDNA labeling technologies have overcome many of the requirements for large amounts of starting material making pooling unnecessary. Ultimately, the number of replicates required will depend on the variability of gene expression in the model system and the acceptance rate of false positives. In addition to significance testing, other factors must be considered when analyzing changes in gene expression. One must make a

distinction between the magnitude of differential expression and the significance level associated with a change in gene expression, since without formal proof, there is no way to conclude that a 10-fold change in steady state mRNA level is any more biologically relevant than a 2-fold change. In fact, the value of fold changes themselves can be an inaccurate representation of the underlying changes. For example, if a gene is expressed at an arbitrary level of 50 transcripts per cell, a 2-fold induction would correspond to a net gain of 50 transcripts per cell. However, a 2-fold repression would correspond to only a net loss of 25 transcripts. A loss of 50 transcripts would correspond to an infinite fold-repression. Therefore, net increases or decreases in transcript abundance may need to be considered when interpreting the biological significance of an observed change. In the absence of robust statistical analysis of the data, confidences must rely on secondary verification of gene expression changes using alternative methods, such as RT-PCR or Northern blots. Finally, when trying to interpret the biological relevance of changes in mRNA, one must concede that mRNA levels do not always reflect protein abundance or activity.

Biomarkers are mechanistically-based experimental endpoints that are designed to efficiently detect and characterize chemical exposures. Biomarkers have been frequently used to detect and characterize xenobiotics for hormone-like activity. For example, an early biomarker that was developed for detecting estrogen exposure *in vivo* is the egg yolk protein, vitellogenin (107). This protein is normally expressed in female oviparous vertebrates in response to endogenous circulating estrogen. Its detection in male oviparous vertebrates,

however, is diagnostic of exogenous estrogen exposure since males do not normally express vitellogenin due to their low levels of endogenous estrogen. Both vitellogenin mRNA and protein have been used to detect chemicals with estrogenic activity *in vivo* and in cultured hepatocytes (108-112). Many biochemical, hormonal and physiological endpoints are also used to screen for potential EEDs in rodents, including agonists and antagonists of estrogen, androgen, progesterone, dopamine and thyroid hormones (113).

Despite the wide use of screening assays for EEDs, they remain costly, time consuming, and only identify a limited spectrum of biological activities. By contrast, molecular biomarkers such as mRNA provide a more rapid and sensitive marker that can detect a wider variety of biological activities. Microarrays provide a powerful tool to identify a larger number of molecular biomarkers of exposure to EEDs and other xenobiotics. The first example of microarrays being used to study hormonal gene regulation *in vivo* was by Feng et al. (114), who used fluor-based microarrays to study hepatic gene expression in hypothyroid mice treated with or without thyroid hormone (T_3). Hypothyroid mice were used to provide a model system with low levels of endogenous T_3, thus increasing the sensitivity to detect subtle changes in gene expression in response to T_3 treatment. They were able to verify changes in the expression of genes previously known to be affected by T_3, as well as identify many novel genes that were both positively (14 genes) and negatively (41 genes) regulated by T_3. This approach could also be utilized for other hormone agonists and antagonists. With new knowledge of biomarkers of exposure to a large number

of hormone agonists and antagonists, higher throughput assays can now be utilized to screen chemicals for a number of potential endocrine modulating activities *in vivo*. To increase their predictive value, these biomarkers must be validated in other tissues or other *in vivo* and *in vitro* model systems. In addition, the establishment of potential biomarkers should be correlated to adverse physiological effects, since changes in gene expression do not always imply toxicity.

Microarray-based screening is also valuable when studying xenobiotics with unknown mechanisms of action, since identifying novel biomarkers associated with toxicity can often give valuable insight into mechanisms of action and may explain pathogenesis. For example, Holden et al. (115) used radiolabel-based microarrays to discover genes associated with hepatotoxicity. In their model system, cultured human hepatoma cells (HepG2) were exposed to carbon tetrachloride (CCl_4) for 8 hours. Gene expression was compared to cells treated with dimethyl formamide, a chemical not implicated in hepatotoxicity, rather than vehicle solvent. While 47 genes were changed in expression, interleukin-8 (IL-8) was further studied by northern blot and ELISA. The increase in both IL-8 mRNA and protein expression was found to correspond to the time-dependent decrease in cell viability, thus implicating IL-8 in hepatotoxicity. However, it is unclear whether IL-8-induction causes cell damage, or is induced in response to cell damage. Therefore, it would be of interest to determine if the response of IL-8 is specific to CCl_4 or if its expression is associated with other hepatotoxicants. Microarrays have also been used for identifying human genes

responsive to other physical and chemical stresses, such as irradiation-induced apoptosis (116,117), DNA-damaging agents and anti-inflammatory drugs (118), metals, and combustion by-products (119-121).

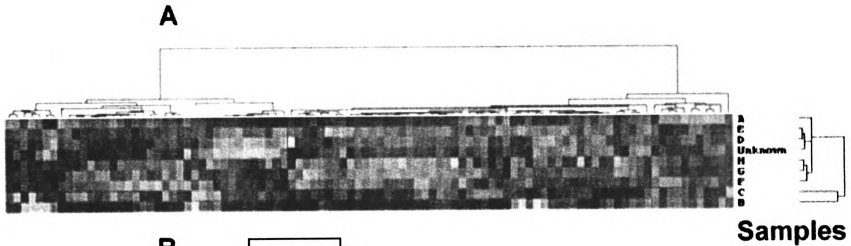
Ultimately, one would like to use biomarkers to monitor exposure in human populations; however, transcriptional responses to chemical stressors in human cells are complicated by cellular heterogeneity and genotype, which present further challenges to interpreting gene expression changes in human populations. Another potential drawback of gene-based biomarkers is their lack of specificity, since genes are often regulated by more than one signaling pathway, and failure of a biomarker to respond to exposure does not necessarily indicate absence of effect. Having multiple markers of exposure that are correlated with adverse effects, in addition to replication and secondary verification of microarray results, are vital for identifying robust predictive biomarkers of EEDs and other xenobiotics.

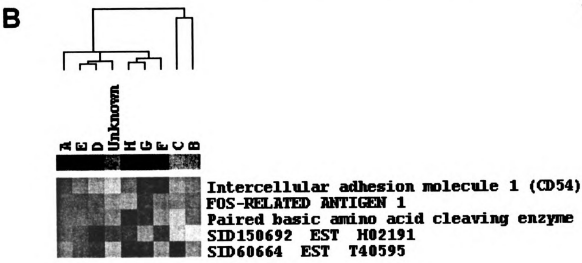
Multi-conditional expression analysis

Measuring changes in gene expression over time or across increasing doses provides information on the kinetics and coordination of gene expression during the dynamic processes of cellular homeostasis. Analyzing gene expression data across multiple samples can also reveal underlying similarities among different conditions, thus producing correlates of gene behavior that can be used to predict and diagnose cellular responses to exogenous chemicals. In order to extract ordered subsets of information from disordered sets of multi-conditional gene expression data, a number of multivariate methods have been

applied. The most common method for clustering multi-conditional gene expression data is hierarchical clustering, which was made popular by the work of Eisen et al. (122). Principal component analysis (123) and partitioning methods, such as k-means clustering (124) and self-organizing maps (SOMs) (125) have also been applied to multi-conditional gene expression data sets. In general, these methods attempt to find order among disordered data sets by grouping similar objects together. Grouping genes based on similarity of expression across multiple conditions is desirable for a number of reasons. For example, two genes of similar expression characteristics may be coordinately regulated and therefore involved in a similar function and/or under the same regulatory control. In addition to grouping genes, samples can be clustered based on the expression of all genes on the microarray. Gene expression profiles induced by chemicals that share a similar mechanism of action can be correlated under certain conditions (Figure 2) (106,118,126). Therefore, it should be possible to predict a potential mechanism of action for a chemical of undefined toxicity based on correlated expression to chemicals of known mechanism. This approach can be used to identify potential EEDs based on large-scale comparison of expression profiles induced by chemicals of known mechanism, such as estradiol, testosterone, T_3, and other hormones that modulate the endocrine system. The overall process of hierarchical cluster analysis begins with defining the objects to be clustered by a measure of similarity. The Pearson correlation coefficient is a common metric that quantifies how well two variables vary together.

Figure 2. Hierarchical cluster analysis of genes and samples. A) This hypothetical data set demonstrates how hierarchical clustering of both genes, across the horizontal axis, and samples (i.e. array experiments), along the vertical axis, illustrates the relationship between gene expression and different experimental conditions. Each column represents a gene and each row is a single microarray experiment measuring gene expression for 100 genes. In this example, each of the nine rows represent an experiment comparing an untreated sample to a sample treated with one of nine different chemical, including 8 chemicals of known mechanism (labeled A to H) and one chemical of unknown mechanism. Genes are hierarchically clustered to indicate the similarity between genes in their expression profile across the nine samples. The length of each branch indicates the relative similarity between branches of the tree. Likewise, the samples are also hierarchically clustered to indicate similarity in expression profile induced by each of the nine chemicals. From the sample dendrogram in A, the expression profile induced by the unknown sample appears most similar to the expression profile induced by chemicals D and E. Note that the similarity between the unknown and chemicals D and E are the same, since both D and E can be reordered about their common node. Therefore, the unknown chemical may act with a similar mechanism of action as D and E. The pink colored branch of the tree is highlighted in B. B) A close up of a cluster within the dendrogram indicates the genes within that cluster and their pattern of expression across the nine samples. C) The color hue and intensity represents the fold change in gene expression, as indicated in the color bar. Green indicates repression of gene expression in the test sample relative to the control sample. Red indicates induction of gene expression in the test sample relative to the control sample. Black indicates no change (i.e. ratio of 1) and grey indicates no data point available for that gene on its respective array.


A
A
E
D
Unknown
H
G
F
C
B
Samples


B
A
E
D
Unknown
H
G
F
C
B
Intercellular adhesion molecule 1 (CD54)
FOS-RELATED ANTIGEN 1
Paired basic amino acid cleaving enzyme
SID150692 EST H02191
SID60664 EST T40595


C
Fold repression
Fold induction
>8 >6 >4 >2 1:1 >2 >4 >6 >8

The correlation coefficient *r* is always between –1 and 1, where 1 indicates an identical profile, 0 indicates the two profiles are independent, and –1 indicates the profiles are inversely related. The advantage of this statistic is that it captures similarity in shape without emphasis on magnitude as it is invariant to scale. Correlation measures are often transformed into Euclidean distances prior to clustering. This transformation accounts for similarity with all other genes, rather by than a single pairwise comparison. It is also less sensitive to random fluctuations in expression measurement such that two genes that exhibit poor pairwise correlation may still be similar by virtue of their correlation with all other genes. A matrix of pairwise distances is clustered and visualized using a dendrogram, which is similar to a phylogenetic tree. To begin clustering, each object (i.e. gene or sample) is represented by a single cluster. The two most similar objects are then merged into a new pseudo-object. A new distance measure is calculated for the pseudo-object and merged with the next most similar object. This process is repeated in an iterative fashion until only one pseudo-object remains, which represents the root of the tree. By the process of clustering, objects are organized into branches, such that branches of the tree that are adjacent are more closely related, and the length of the tree branch reflects the degree of similarity between objects. Finally, the branches of the tree are ordered and colored to indicate graphically the nature of the relationships within and between the branches (Figure 2).

Both the genes and the experimental conditions can be hierarchically clustered in a two-dimensional dendrogram. In this manner, treatment conditions

that induce similar expression profiles across all genes on the microarray are closer together on the tree in the first dimension, and presumably mechanistically related, while genes that are similar in expression profile across all conditions are closer together on the tree in the other dimension, and perhaps functionally related. This approach can be used identify treatment conditions and to illustrate relationships between multiple conditions and gene expression so that subsets of genes, rather than a single biomarker, can be used as predictors of cellular response to EEDs and other xenobiotics.

Hierarchical clustering has been suggested to be inappropriate for gene expression data since phylogenetic trees, or dendrograms, are best applied to situations of true hierarchical descent, such as evolution. However, gene expression does not follow a hierarchy but rather is characterized by distinct mechanisms. When hierarchical clustering is applied, the data is forced into associations at some level, regardless of the relationship between the data. As two branches of the tree are joined into one, they become less similar and eventually meaningless. As an alternative to imposing a hierarchical descent on the data, partitioning methods, such as SOMs and k-means clustering, can divide data set into similar, but distinct groups. Hierarchical clustering can then be applied to each partitioned set of genes.

One of the first applications demonstrating the utility of gene expression profiling to characterize mechanisms of toxicity was by Marton et al. (126). Using cDNA microarrays containing essentially every open reading frame (ORF) in the yeast genome (6000+), Marton et al. were able to show that the gene expression

profile induced by the immunosuppressant FK506 was highly correlated with the expression profile induced by the mechanistically similar immunosuppressant cyclosporin A. These expression signatures were not, however, correlated with the expression profile induced by other unrelated drugs. These experiments demonstrate the principle that chemicals can induce characteristic and unique gene expression profiles. Therefore, expression profiles may be used to identify and classify unknown chemicals with respect to mechanism of action. The use of yeast as a model system allowed Marton et al. to also verify the drug target (i.e. calcineurin) by correlating the expression profile of the immunosuppressants to the expression profile induce by genetic disruption of the target protein. It was also demonstrated that off-target effects exist for FK506 since a distinct profile of gene expression was produced in a calcineurin mutant strain treated with the immunosuppressant. Although the toxicity of FK506 is primarily mechanism-based, these effects point to potential causes of unwanted side effects.

The studies by Marton et al. support the use of expression profiling to distinguish mechanisms of action of unknown EEDs, even in simple model systems. However, a large reference database of expression profiles induced by a number of diverse hormones and other EEDs of known mechanism is required. The utility of this approach has been demonstrated in yeast. Using a 'compendium' of yeast expression profiles from over 300 diverse mutants and chemical treatments, Hughes et al. (106) were able to identify a previously unknown drug target for the commonly used topical anesthetic dyclonine. This was accomplished by matching gene expression profiles caused by

uncharacterized perturbations (e.g. gene disruption or chemical inhibition) with a large set of reference profiles corresponding to disturbances of known cellular pathways. Hierarchical clustering and correlation measures were used to match similar expression profiles. When the dyclonine-induced expression profile was compared to the compendium, it was found to be correlated ($r = 0.82$) with the profile resulting from genetic disruption of the ergosterol pathway, specifically *erg2*. The human gene with the greatest sequence similarity to the erg2 protein was found to be the sigma receptor, which is known to bind a number of neuroactive drugs and other inhibitory compounds that target both erg2p and the sigma receptor. Despite the use of yeast as a model system, a potential mechanism of action for dyclonine can be inferred for mammalian systems.

Gene expression profiling could also be extended to identify regulatory motifs, such as hormone response elements, that govern the response to hormones and other EEDs. For example, Gasch et al. (127) used yeast to identify regulatory motifs that play a role in the transcriptional response to a variety of physical and chemical perturbations, including heat shock, osmotic shock, nitrogen and amino acid depletion, oxidative stress, and others. When the gene expression profiles induced by a panel of environmental stresses were compared by hierarchical clustering, a set of ~900 genes were found to show a similar response to almost all of the environmental changes. Their regulation, however, was dependent on many signaling systems that acted in a condition-specific and gene-specific manner, rather than being controlled by a single stress-sensing pathway. To determine what factors governed the response to

stress, the promoters of subclusters of stress-responsive genes were analyzed for common regulatory elements. It was found that many stress-responding genes contained Msn2 and/or Msn4p binding sites. By examining the expression profile of yeast mutants null for *msn2 msn4*, or over expressing MSN2 or MSN4, it was determined that these transcription factors play a role in regulating expression of a subset of responsive genes following environmental stress. Other responsive genes were not affected by MSN2/4 and are thought to be under control of other independent signaling pathways. These elaborate set of studies demonstrate the complexity at which cells can detect and respond to unique forms of physical and chemical stressors, and underscores the utility of transcriptional responses to diagnose cellular perturbations and decipher the mechanisms of action of chemicals.

Challenges and limitations of gene expression profiling in mechanistic and predictive toxicology

Toxicogenomics is expected to accelerate drug development and aid risk assessment. Recent experiments applied to cancer genetics have demonstrated the potential of gene expression profiling to accurately classify disease phenotypes (100,128), thus lending hope that expression profiling may classify and thus predict phenotypes of toxicity. Despite these expectations, it is still uncertain how gene expression profiling experiments will ultimately contribute to our understanding of toxicity and allow us to realize the full potential of this new technology. Although there has been much review and hyperbole surrounding the potential applications of toxicogenomics, these novel and unverified

approaches to toxicological problems require an awareness of the constraints of the methodology in order to design and interpret gene expression profiling data. Pennie et al. (129) have also discussed the possibilities and caveats of gene expression profiling in the context of mechanistic and predictive toxicology and have addressed the certainty, biological relevance, and the need for validation of microarray data. The purpose of this review chapter is to illustrate the current constraints of gene expression profiling in mechanistic and predictive toxicology, and to stress how current experimental designs may confound accurate interpretation of genome-scale data. The limitations described are not intended to discourage the application of gene expression profiling technologies to mechanistic or predictive toxicology, but rather guide experiments that will produce more interpretable and useful data.

Gene expression profiling in mechanistic toxicology –a hypothesis generating tool

There is a certain degree of faith that gene expression profiling will reveal the mechanisms of action of chemicals and drugs despite the inherent limitation of genomic and proteomic experiments which measure single endpoints (i.e. RNA or protein levels), albeit for thousands of genes at a time. Consider the many experiments and endpoints that have been employed to explain the mechanism of action of some previously characterized chemicals and drugs. It is understood that mechanisms of action are far more complex and affect more than simply the levels of cellular macromolecules. Many toxicants affect enzyme activity, DNA integrity, redox status, membrane integrity, and other processes

that are not amenable, as yet, to genome-wide measurements. Although alterations in the above processes are likely to indirectly affect the expression of genes and proteins, the question remains how do we extrapolate a mechanism of action from the one endpoint. Similarly, predictive toxicology attempts to infer the potential mechanism(s) of action of an unknown agent on the basis of correlation to large databases of activity or expression profiles (106,128,130).

Can mechanisms of action be determined or predicted from gene expression profiling? To answer this question we need to first define what is meant by a mechanism of action, since the term is often used with many connotations. The mechanism of action of a chemical or drug is described by the series of molecular events following interaction of a chemical with its cellular target(s) and the subsequent alteration(s) in target function that precedes a cascade of cellular events that ultimately leads to the observed effect. The challenge of trying to determine the mechanism of action from measuring steady state mRNA or protein levels is that many toxicants and drugs initiate toxicity by binding to proteins and/or altering macromolecules (although with exceptions, as noted below) and not by directly inducing gene expression or altering gene product stability or turnover. For example, the mechanism of action of acetominophen (APAP)-induced hepatocellular necrosis is due to cytochrome P450-catalyzed activation of APAP to the electrophilic NAPQI intermediate, leading to arylation and thiol oxidation of cellular proteins. These events in turn lead to non-specific and/or undefined alterations in protein function and subsequent changes in nuclear and organelle structure and function leading to

irreversible cell injury and oncotic necrosis (131). The mechanism of action of APAP has been delineated through many detailed chemical and biochemical experiments that could not have been revealed through observation of gene expression changes alone.

This is a limited view of the complete spectrum of toxic effects initiated by APAP as it has been observed to cause chromosomal aberrations, apoptotic DNA fragmentation, unscheduled DNA synthesis, oxidative stress, altered calcium homeostasis and inhibition of cell proliferation (132); and references therein). The fact that multiple cellular signaling pathways may converge to alter the expression of the same gene products also makes it difficult to identify the affected pathway from observing gene expression changes. The above arguments illustrate the point that most chemicals and drugs will act through multiple mechanisms of action that will depend on dose, timing and duration of exposure, and cell phenotype. Each individual mechanism represents an initiating event which is by itself inadequate to drive progression of toxicity, but that together act in concert to cause cell injury and/or death. Although gene expression would be expected to be altered as a result of APAP exposure, the changes in gene expression will reflect secondary outcomes due to primary upstream events starting with the interaction of APAP with its target protein(s). Therefore, our ability to define the mechanism of action of a compound using gene expression profiling technologies will be highly limited in resolution. In the best case scenario, gene expression changes in cellular perturbation experiments will lead to many new testable hypotheses that will require

subsequent molecular and biochemical experiments to reveal and confirm precise mechanisms of action.

Ultimately, being able to define the mechanisms of organismal toxicity will depend on our understanding of cellular and tissue level effects and how they are related to the molecular changes in target cells. Since changes in gene expression do not necessarily imply toxicity, gene expression profiling experiments need to be integrated into larger studies that examine multiple endpoints at the molecular, cellular, tissue and physiological levels in the context of the whole organism. As noted (129), this creates a further challenge in trying to integrate knowledge at all levels of biological organization and highlights the need for an interdisciplinary approach in mechanistic toxicology.

In some instances of toxicity, a direct and primary response affecting gene expression and subsequent initiation of toxicity, due likely to a receptor-mediated pathway, may be used to explain the mechanism of action of chemicals, including nongenotoxic carcinogens or endocrine disruptors. This will be particularly true for therapeutics and drug candidates as it has been estimated that close to 50% of marketed drugs act through receptors (133). These observations will of course be complicated by parallel mechanisms of toxicity which may or may not be receptor-mediated, yet may augment the receptor-mediated events. The challenge will be to distinguish the therapeutic affects from the pathological changes. This will require establishing time-dependent relationships between dose and toxicity, which may or may not be linear. Where alterations in gene expression precede or coincide with toxicity, our ability to

understand the mechanism of action will be limited to our understanding of the pathways that regulate transcription of the affected genes and their kinetics of expression. This is currently a major limitation in understanding why a particular gene or cluster of genes is observed to be up- or down-regulated, since only a small fraction of the estimated 100,000+ human genes have been studied at the level of transcriptional regulation. Combining the identification of gene regulatory elements with expression profiles in microarray experiments (134) represents an industrious approach to begin to understand what transcription factors and upstream signaling molecules are governing the observed response in gene expression following chemical or drug exposure.

Gene expression profiling has possibly a greater potential to reveal modes of action through the analysis of secondary responses and/or the series of contingent regulatory events induced by chemical or drug exposure. The mode of action of a chemical or drug can be described, in part, by a fundamental obligatory step directing toxicity, or adverse cell fate, be it reversible cell injury, apoptotic or oncotic necrosis, or malignant transformation. Farr and Dunn (102) have noted that organismal manifestations of toxicity can be explained by combinations of a limited number of cellular outcomes from a limited number of cell and/or tissue types. Furthermore, multiple mechanisms of action may converge at common points to trigger the same molecular response. If this is true, then the number of possible modes of action will be limited to the number of molecular responses that can drive the obligatory step towards a discrete cellular outcome. It follows then that gene expression profiles cannot be used as an

explanation or predictor of toxicity unless correlated with a toxic endpoint. Again, this underscores the need to integrate genomic experiments with experiments examining effects at higher levels of biological organization that are intended to assess toxicity in the context of the whole organism. By understanding the gene expression changes that direct a unique cellular outcome (i.e. the mode of action), we can begin to use gene expression profiles to explain and potentially predict toxicity.

Predictive toxicology – fact or fiction?

It has been proposed that each chemical that acts through a particular mechanism of action will induce a unique and diagnostic gene expression profile under a given set of conditions (96). Indeed, proof-of-principle experiments in *S. cerevisiae* have revealed that the response to inhibitory compounds mimics the loss of function of its target or pathway for at least 6 compounds (106,126) and references within). For example, genetic disruption of calcineurin in *S. cerevisiae* resulted in a gene expression profile highly correlated with the expression profile of wild-type cells treated with FK506 or cyclosporin, antagonists of the calcineurin signaling pathway. To estimate the significance of the relationship, the FK506-treatment profile was compared to more than 40 randomly selected deletion strains or drug-treated cells and found to be uncorrelated (126). Whether predictive patterns in gene expression can be observed in mammalian systems remains to be shown, although preliminary studies suggest they can (135,136). Therefore, there is significant potential for chemicals and drugs to be classified based on the similarity of their induced gene expression profile by comparison

with expression profiles induced by chemicals or drugs with known mechanism of action using multivariate statistical methods and correlation metrics. In some cases, however, their classification may be limited to the affected signaling or metabolic pathway rather than by target protein in the pathway. By extension of this observation, gene expression profiles are anticipated to produce knowledge of a subset of commonly regulated genes that can be used as biomarkers to predict modes of action.

While it has been pointed out that the number of possible patterns of differential gene expression, even when expressed as binary variables, is enormous (102), subtle differences in the number and magnitude of gene expression changes have proven to be sufficient to classify expression profiles into distinct clusters when applied to *S. cerevisiae* (106). The utility of this approach, however, may be lost when outside the context of a large database, or compendium, of expression profiles since subtle changes in relative expression level (i.e. less than 2-fold) are usually considered unreliable in isolation (106). Based on gene expression profiles of yeast mutants, it has been estimated that there exists 300 to 700 distinct full genome transcriptional patterns from a full set of 5000 yeast deletion mutants profiled under a single condition (106). Although this was a crude prediction, an extrapolation to mammalian systems may predict substantially more distinct transcriptional patterns under a single condition. Classifying transcriptional responses into distinct diagnostic clusters may prove more problematic if responses under different conditions do not extrapolate under different conditions. For example, transcriptional responses may differ

between one target cell to another, from cell culture to *in vivo* conditions, or from rodent models to humans. Thus, the predictive power of gene expression profiling may be limited to the model system employed and the prototypical compound with known mechanisms used to generate the diagnostic expression profile. As yet there is no published data to support that predictive expression profiles will extrapolate to other tissues or *in vivo* settings.

The challenge of interpreting gene expression data

Currently, there is a significant knowledge gap in our understanding of the molecular events that govern toxicologically relevant outcomes. In any event, the changes in gene expression directing cell fate will reflect, in part, an active physiological response that is non-toxic. These responses may include, but are not limited to, host-defense responses (e.g. acute phase proteins, cytokines, DNA repair enzymes), adaptive responses (e.g. hyperplasia, metaplasia, hypertrophy, atrophy), and regenerative or protective responses (proliferation, differentiation). In addition, there will be secondary responses following toxicity that will reflect pathology as a result of disturbances in cell function. These responses are likely to be idiosyncratic and diverse across cell types due to the interaction of pathological responses with the physiological mechanisms of detoxification and repair that are cell-specific. Again, the challenge then lies in differentiating the physiological responses from the diagnostic pathological changes in light of confounding experimental artifacts inherent in the model system and the experimental design.

Consider, for example, an experiment designed to measure time- or dose-dependent changes in gene expression following an EC_{50} dose of a cytotoxic chemical in cultured cells. When administering a dose that kills half the cell population, the measured response (i.e. mRNA or protein level) in the affected culture will be a combination of multiple factors, including the gene expression changes in dying cells due to treatment, adaptive changes in surviving cells due to treatment, and normal responses in living cells due to adjacent necrotic cells. This would be particularly relevant *in vivo* since necrosis can induce a regenerative or inflammatory response in some populations of unaffected or resistant cell types. The heterogeneous responses are likely to be highly dependent on the tissue- or cell-type affected, again highlighting the limitation of extrapolating one model system to another. Measuring gene expression changes following sublethal exposure concentrations may be more likely to reveal treatment-induced changes that initiate toxicity before heterotypic cellular responses obscure interpretation. This will require a complete characterization of the full dose- and time-response relationship including a qualitative description of cellular changes as correlates.

Artifactual complications may also apply to other classes of chemicals, particularly chemicals that act through receptor-mediated pathways since receptor expression is usually restricted to discrete cell types. Subsequent changes in paracrine signaling may have dramatic effects that could lead to misinterpretation of gene expression profiles in cultured cells. This would also be particularly relevant *in vivo* where cellular complexity plays a dominant role in

adaptation and defense, or when target tissues are affected secondary to primary targeting of a proximal endocrine gland, such as the pituitary or thyroid. Furthermore, when analyzing gene expression profiles from whole tissues as part of a whole animal toxicology study, the relevant gene expression changes in the specific cell types targeted by chemical or drug may be masked or diluted by the benign changes in surrounding cell types. For example, consider the cell-type specific toxicity of alloxan or streptozotocin on the β cells of the pancreas and the fact that the β cells represent less than 2 % of the pancreatic cell population. The ability to detect changes in gene expression within 2 % of an RNA sample derived from whole pancreas is likely below the limits of sensitivity of current genomic profiling platforms. Compensatory changes in other, more abundant, cell types may also negate any changes in the targeted cell and could even result in the opposite conclusion regarding message or protein abundance. Being able to measure gene expression profiles in individual targeted cells or cell types, by using laser capture microdissection for example, would be more desirable in these instances (137,138). However, prior knowledge of the target tissues and/or cell types from pathology studies is typically required for this level of investigation. This would preclude its utility in higher throughput predictive assays that are currently desired, but would prove useful for mechanistic studies. Reducing the number of observations (i.e. gene expression profiles) and correlating them with a binary response (i.e. apoptosis, DNA damage) may allow for the identification of a more robust set of predictive markers with utility in higher throughput systems. Realization of such a scenario will be heavily

dependent on the standards of known modes of action that are available, the reproducibility of the model system, statistical robustness of the data and the application of multivariate methods of analysis to reduce the data set into a comprehensible and manageable number of components for purposes of classification.

Other limitations to consider arise when adverse cellular or tissue functions are observed in the absence of discrete cellular outcomes, such as cell injury or death. In these instances, subtle changes in cell function, such as reduced responsiveness to endocrine signals or altered secretion or production of signaling molecules, will be more difficult to observe since relevant changes may be transient, posttranslational and/or in non-target organs. Many endocrine disruptors will likely fall into this category. Perhaps the greatest source of complexity and variability in gene expression profiling experiments *in vivo* stems from non treatment-related phenomena, or intrinsic variability, which is difficult, if not impossible to control and reproduce. Normal fluctuations in gene expression will occur as a result of differences in age, gender, temperature, light, diet and hormonal status. While age, gender, and the external environment can be tightly controlled within experiments, comparisons between laboratories using similar treatment protocols may be more challenging when environmental factors are not strictly adhered to. Differences in nutritional or hydration status, time of last meal, hormonal fluctuations during estrus, and seasonal and light-induced changes in hormone levels are more difficult to control within experiments. Such intrinsic variation is likely to interact with timing, duration and frequency of

treatments to alter the observed response in gene expression. Like any experiment designed to test a hypothesis, there must be sufficient replication to assure certainty in the experimental results.

The expectations that toxicogenomics will enable us to define mechanisms of action and predict toxicity of unknown agents are supported by recent studies in lower eukaryotes. However, our current ability to define a mechanism of action or accurately predict toxicity in mammalian systems is still in its infancy. Incorporating genomic experiments into larger studies designed to assess effects at higher levels of biological organization is a must if one is to begin to understand and predict organismal outcomes and possibly incorporate gene expression data into mechanism-based risk assessment. The progression of expression profiling into whole animal studies also presents a higher level of complexity that challenges are understanding of biological systems and the interpretation of what changes in gene expression are relevant. It is expected that experience and interdisciplinary collaborations will continue to advance the utility of gene expression profiling in mechanistic and predictive toxicology, however, continued discussion, debate, and the sharing of knowledge and data is vital for toxicogenomics to move rapidly ahead.

CHAPTER 4

EFFECTS OF GESTATIONAL AND LACTATIONAL EXPOSURE TO AROCLOR 1242 ON SPERM QUALITY AND *IN VITRO* FERTILITY IN EARLY ADULT AND MIDDLE-AGED MICE[2]

ABSTRACT

The objective of this study was to examine the effects of gestational and lactational exposure to Aroclor 1242 (0, 10, 25, 50, and 100 mg/kg-bw). Doses were administered to C57BL6 female mice orally every two days from two weeks before mating, during mating, and through gestation until postnatal day 21. Male B6D2F1 offspring were examined for anogenital distance, organ development, epididymal sperm count, sperm motility, and in vitro fertility at 16 and 45 weeks of age. Stomach samples of pups nursing from PCB-treated mothers in the 50 mg/kg dose group were analyzed for PCBs and chlorobiphenylols by high resolution gas chromatography coupled with low resolution mass spectrometry. It was estimated that the nursing pups were exposed to 0.2, 0.6, 1.2, and 2.4 mg/kg/d total PCBs in the 10, 25, 50, and 100 mg/kg dose groups, respectively. This exposure level approaches the maximum FDA recommended levels for PCBs in food and breast milk. The composition of the PCBs in the stomach

[2] Published in Fielden M.R., Halgren R.G., Tashiro C.H.M., Yeo B.R., Chittim B., Chou K., Zacharewski T.R. (2001) Effects of gestational and lactational exposure to Aroclor 1242 on sperm quality and *in vitro* fertility in early adult and middle-age mice. Reproductive Toxicology. 15: 281-292.

samples was different from the parent mixture, as there was a higher proportion of heavily chlorinated congeners, as well as chlorobiphenylols. Anogenital distance at weaning, and liver, thymus, and testes weight at 16 and 45 weeks of age were not affected by PCB exposure. Epididymal sperm velocity and linearity were significantly increased in the 25 mg/kg dose group at 16 weeks of age. Sperm count was increased by 36% in this dose group ($P = 0.06$). By 45 weeks of age, average sperm count in this dose group was similar to that of controls. With the exception of the 50 mg/kg dose group at 16 weeks of age, sperm fertilizing ability in vitro was significantly decreased in all PCB-exposed groups at 16 and 45 weeks of age. These results suggest that fertility in the adult mouse is susceptible to developmental exposure to Aroclor 1242 and is independent of testis weight or epididymal sperm count.

INTRODUCTION

Polychlorinated biphenyls (PCBs) are a class of lipophilic, persistent synthetic chemicals that exist as complex mixtures in environmental and human matrices, including blood, adipose tissue, breast milk, and fetal tissue (139). PCBs are considered potential endocrine disruptors, among many other effects, due to their ability to act as estrogens, antiestrogens and goitrogens (reviewed in (140)). There is concern that exposure to PCBs and other organohalogens may impair male fertility (141). Recent epidemiological evidence suggests that prenatal exposure to PCBs and polychlorinated dibenzofurans can cause adverse effects on semen quality (142). There have also been numerous, but inconsistent, reports of adverse effects on male reproduction following prenatal or postnatal exposure to PCBs in laboratory rodents. These effects include alterations in testis weight, seminal vesicle weight, ventral prostate weight, reduced serum testosterone levels, and impaired fertility (143-149).

The effects of developmental exposure to PCBs on testis weight and fertility in laboratory rodents depend on the test congener or mixture, the dosage, the developmental stage during exposure, and the age of the animal at the time of examination, as well as species and strain. For example, Sager (143) previously showed that male Holtzman rats exposed to Aroclor 1254 through early lactation (from birth to day 9) exhibited decreased fertility at 18 weeks of age and increased testis weight at 23 weeks of age. The decreased fertility was not accompanied by a decrease in epididymal sperm count or changes in sperm

morphology or motility, but rather a decline in the ability of sperm to fertilize eggs (144,146). Cooke et al. (147) demonstrated that neonatal exposure (from birth to day 25) of Sprague-Dawley rats to Aroclor 1254 and 1242 increased testis weight and daily sperm production at 19 weeks of age. These effects appear to be due to PCB-induced hypothyroidism since thyroxine replacement attenuated the increase in testis weight and sperm production in Aroclor 1242-treated rats (147). In contrast to the reduced fertility of Aroclor 1254-exposed pups reported by Sager et al. (143,144,146), all Aroclor 1242-treated pups successfully impregnated females (147). These PCB-induced effects on sperm production and fertility also appear to be independent of changes in serum FSH or testosterone concentration, testicular histopathology, or sperm morphology or motility (146,147).

Previous studies investigating the reproductive effects of developmental exposure to PCBs in rodents have focused on effects in early adulthood, while few studies have addressed whether rodents at later stages of adulthood exhibit the same effects or recover to control levels. Previous studies have observed that neonatal exposure of male B6D2F1 mice to Aroclor 1254 does not adversely affect sperm fertilizing ability *in vitro* until 45 weeks of age (150). Therefore, the objectives of this study were to determine if gestational and lactational exposure of B6D2F1 mice to Aroclor 1242 can cause alterations in organ development and sperm quality and fertility in young adult (16 weeks of age) male offspring and to determine if the effects persisted into middle age (45 weeks of age). To estimate lactational exposure, stomach samples from pups nursing on PCB-treated

mothers in the 50 mg/kg dose group were analyzed for PCBs and chlorobiphenylols (OH-PCBs) using high resolution gas chromatography (HRGC) coupled with low resolution mass spectrometry (LRMS).

MATERIALS AND METHODS

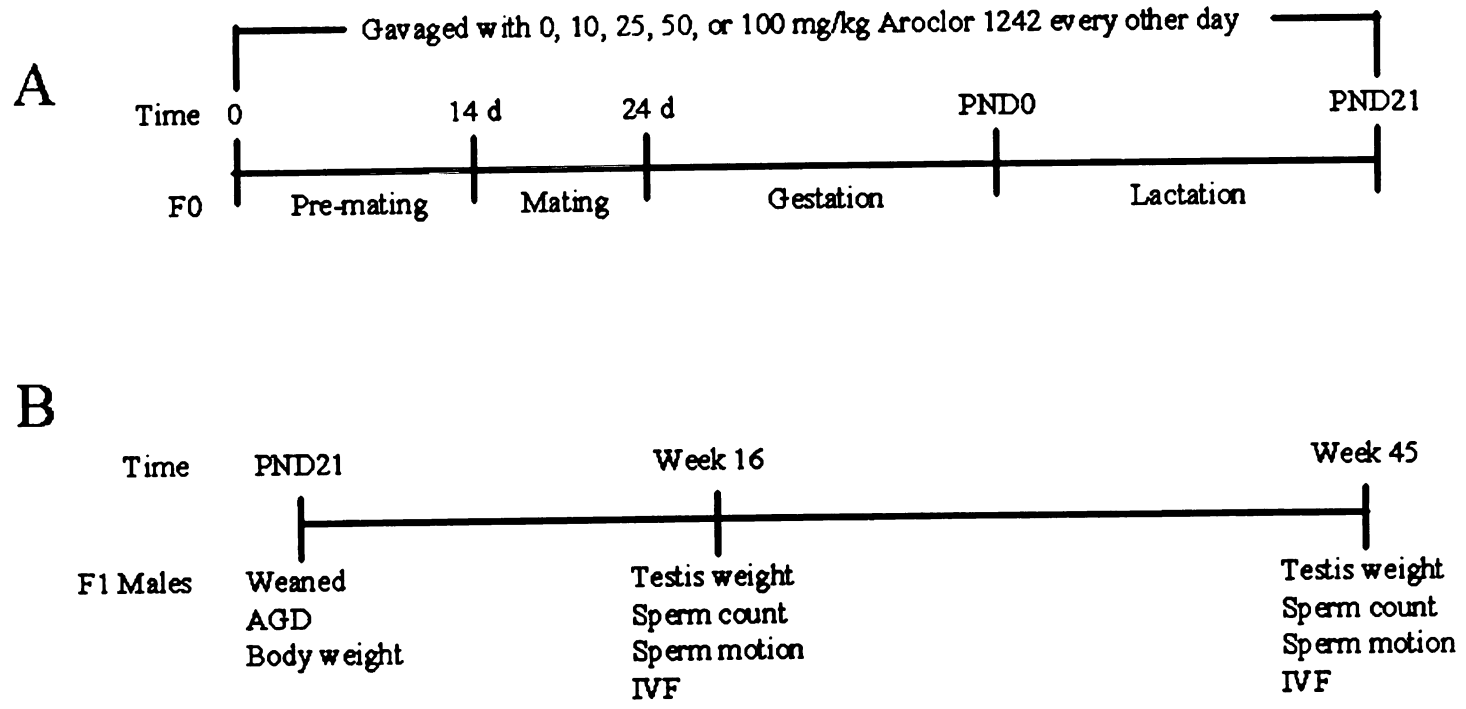
Animals

C57BL6 female and DBA/2 proven breeder male mice were obtained from Charles River Laboratories (Raleigh, NC) and housed in polycarbonate cages with cellulose fiber chips (Aspen Chip Laboratory Bedding, Northeastern Products, Warrensberg, NY) as bedding and maintained in a humidity (30 to 40 %) and temperature (23ºC) controlled room on a 12-h light-dark cycle. All mice receiving PCB treatment were housed in a HEPA-filtered rack in the same room. All animals were given free access to deionized water and rodent feed (Harlen Teklad 22/5, Madison, WI).

F0 Treatment

Fourteen-week-old C57BL6 female mice (F0) were randomly assigned to treatment groups, and treated for two weeks prior to mating, and throughout mating, gestation, and lactation until offspring were weaned on postnatal day (PND) 21 (Figure 1A). Treatment was by gavage every other day with 0.1 ml corn oil (CPC International Inc., Englewood Cliffs, NJ) with or without Aroclor 1242 (S. Safe, Texas A&M, College Station, TX) for a nominal dose of 0 (n=16), 10 (n=9), 25 (n=10), 50 (n=11), and 100 (n=11) mg Aroclor 1242 per kg maternal body weight. There was a 1-d interruption of treatment on the day of parturition (PND 0). The dose of test chemical was adjusted to body weight for each mouse before daily dosing.

Figure 1. Treatment schedule for F0 mice and necropsy schedule for F1 male offspring. A) F0 C57BL6 mice were dosed by gavage every other day with 0.1 mL of corn oil with or without Aroclor 1242 for a nominal dose of 0, 10, 25, 50 and 100 mg/kg of maternal body weight. F0 mice were treated for two-weeks prior to mating with a DBA/2. Treatment continued throughout a maximum ten-day mating period, and through gestation and lactation until PND 21 when the pups were weaned.. B) Male offspring were weaned on PND 21 and housed with same-sex littermates. F1 males were split into two groups and necropsied at 16 or 45 weeks of age. Males were examined for testis weight, sperm count and motion analysis, and *in vitro* fertilizing ability (IVF).


A
Gavaged with 0, 10, 25, 50, or 100 mg/kg Aroclor 1242 every other day
Time
0
14 d
24 d
PND0
PND21
F0
Pre-mating
Mating
Gestation
Lactation
B
Time
PND21
Week 16
Week 45
F1 Males
Weaned
AGD
Body weight
Testis weight
Sperm count
Sperm motion
IVF
Testis weight
Sperm count
Sperm motion
IVF

For mating, each female C57BL6 was paired for ten days with a DBA/2 proven breeder male mouse. Previous studies have shown that B6D2F1 pups are a suitable model system for *in vitro* fertilization (IVF) studies since B6D2F1 pups are very responsive to superovulation and control eggs exhibit high fertilization rates *in vitro* (148). One or two females were housed per male per cage during the mating period. Females were housed individually for the duration of the study following the ten-day mating period. Females not conceiving or giving birth to live young were euthanized. Offspring were weaned on PND 21 and housed with same sex littermates. At this point, the F1 males from each litter were randomly split into two groups for assessment of sperm count and motion analysis and *in vitro* fertilizing ability (IVF) at either 16 or 45 weeks of age (Figure 1B). F1 females were superovulated within a week of weaning and assessed for oocyte fertilizing ability *in vitro* (data not shown). The mothers were terminated on PND 21.

Reproduction and necropsy

F0 body weight was recorded daily and liver and thymus weights were recorded on PND 21. The number of live pups born was recorded on the day of birth (PND 0). Litter weight was recorded on PND 1. Pup survival and sex ratio for each litter were recorded on PND 21. On PND 21, body weights were recorded and male anogenital distance (AGD; the length of the perineum from the base of the sex papilla to the proximal end of the anal opening) was measured using vernier calipers to an accuracy of 0.8 mm. Body, liver, thymus, and testis weights of F1 mice were measured at necropsy at 16 and 45 weeks of

age. In addition, randomly selected female F1 mice were sacrificed on PND 4 or 5 from the control (n=1), 50 (n=4), and 100 (n=1) mg/kg dose groups. Whole stomachs were collected and individually frozen at –20 ºC in amber vials for subsequent PCB congener analysis.

HRGC/LRMS analysis of PCBs and chlorobiphenylols in pup stomach samples

Individual stomach samples, consisting of both stomach contents and tissue, were transferred to glass culture tubes containing 6 mL of concentrated hydrochloric acid. The tube was capped and sonicated for 20 min and allowed to stand overnight to complete the digestion. The resulting digestate was extracted 3 times with hexane and the extracts combined. For PCB analysis, aliquots of the hexane extracts were spiked with mass-labeled ($^{13}C_{12}$) PCBs and then washed with concentrated sulfuric acid (3 ml) followed by HPLC-grade water (3 ml) and finally 0.1 M aqueous potassium carbonate (0.5 ml). The hexane extracts were then concentrated to 100 µl with solvent exchange into nonane and analyzed by high resolution gas chromatography coupled with low resolution mass spectrometry (HRGC/LRMS) as described below. For analysis of chlorobiphenylols (OH-PCBs), aliquots of the hexane extracts were first spiked with mass-labeled ($^{13}C_{12}$) OH-PCBs and then washed with HPLC-grade water (3 mL). The hexane extracts were then concentrated to 100 µl with solvent exchange into toluene and analyzed by HRGC/LRMS as described below.

Analysis of PCBs in the stomach extracts was performed using a Hewlett Packard 5890 HRGC coupled to a Hewlett Packard 5970 mass selective detector

(MSD). The MSD was operated in the selected ion monitoring (SIM) mode. The ions monitored were those in the molecular ion clusters of mono- through decachlorobiphenyl and their $^{13}C_{12}$ analogues. The capillary column used was a 30 m DB-5 column (J&W Scientific; 0.25 mm i.d., 0.25 µm film thickness) with splitless injection (250 ºC). The HRGC oven temperature program was as follows: initial temperature = 100ºC; initial time = 10 min; temperature program = 10ºC/min; final temperature = 320ºC; final time = 4 min.

Analysis of OH-PCBs in the stomach extracts was performed using a Hewlett Packard 5890 series II HRGC coupled to a VG 70SE high resolution mass spectrometer (HRMS). The HRMS was operated in the EI/SIR mode at 10000 resolution. The ions monitored were those in the molecular ion clusters of the native and mass-labeled OH-PCBs from dichloro to pentachlorobiphenylols. The capillary column used was a 60 m DB-5 column (J&W Scientific) with the injector port at 250ºC and source temperature at 300ºC. The HRGC oven temperature program was as follows: initial temperature = 100 ºC; initial time = 7 min; temperature program = 10ºC/min; final temperature = 320ºC; final time = 11.2 min.

The data was corrected for recovery of the $^{13}C_{12}$-labelled PCB and OH-PCB surrogates. Recoveries of the $^{13}C_{12}$-labelled PCB surrogates ranged from 64% to 123%. Recoveries of the $^{13}C_{12}$-labelled OH-PCB surrogates ranged from 54% to 125%. A 4-point calibration curve was used with the native PCBs (71 individual congeners) ranging in concentration from 0.01 ng/µl to 0.25 ng/µl. The $^{13}C_{12}$-labelled PCB surrogates were at 0.125 ng/µl in each calibration solution.

The PCB surrogates used were PCB 3, 15 , 28, 52, 70, 77, 101, 105, 118, 126, 138, 153, 156, 167, 169, 170, 178, 180, 189, 194, 206, 209. For the OH-PCBs, a 4-point calibration curve was used containing one OH-PCB per congener group (Cl_2 –Cl_5) and one $^{13}C_{12}$-labelled OH-PCB surrogate per congener group. The native OH-PCBs in the calibration solutions ranged from 5 pg/µl to 500pg/µl with the surrogates at 50 pg/µl in each calibration solution. The $^{13}C_{12}$-labelled OH-PCB surrogates used were 3',4'-dichloro-4-[$^{13}C_{12}$]biphenylol, 2',4',5'-trichloro-4-[$^{13}C_{12}$]biphenylol, 2',3',4',5'-tetrachloro-4-[$^{13}C_{12}$]biphenylol, and 2',3,4',5,5'-pentachloro-4-[$^{13}C_{12}$]biphenylol.

Sperm count and motion analysis

Cauda epididymal sperm were collected from F1 males at 16 and 45 weeks of age by excising both epididymides and piercing with a 25 gauge needle in a 1-ml organ culture dish (Becton Dickinson, Franklin Lakes, NJ) containing 1 mL Brinster's BMOC-3 medium (Gibco/BRL, Grand Island, NY), a capacitation supporting medium, supplemented with 3 µg/ml penicillin and 3 µg/ml streptomycin (Gibco/BRL). Sperm suspensions were incubated at 37ºC in a humidified 5 % CO_2 air environment for 30 min before sperm concentration and motion analysis and 60 min before insemination (see below). Sperm suspensions (20 µl) were placed on a 20 µm deep counting chamber and analyzed using a CellSoft computer-assisted digital image analysis system (CASA; CRYO Resources Inc.). A minimum of 100 cells were analyzed from each animal to determine concentration, motility, velocity, linearity, mean amplitude of lateral head (ALH) displacement and beat/cross frequency. Motility

is expressed as the percentage of sperm that move faster than 20 μm/s. Velocity is defined as the average distance (μm) traveled by motile sperm in 1 s. Linearity is the ratio of the straight to actual distance traveled, averaged over all sperm. ALH displacement is a measure of the lateral movement of the sperm head from a computer-calculated mean of its track. The beat/cross frequency (Hz) is the numbers of beats (or crosses) per second. Every time the sperm cell crosses the computer-calculated curval mean, the computer counts that crossing as one beat. All measurements for each animal were performed in duplicates and the average recorded.

***In vitro* fertilization assay**

The *in vitro* fertilizing ability of sperm from 16- and 45-week-old F1 male mice was assessed by inseminating oocytes collected from untreated 3-week-old B6D2F1 female mice that were superovulated with 10 IU pregnant mare's serum gonadotropin (PMSG) (Sigma, St. Louis, MO) followed 48 h later with 10 IU human chorionic gonadotropin (HCG; Sigma). Fourteen to 16 h later the oocytes from each female were collected from the proximal oviducts. Oocytes from each mouse were incubated in a 1 ml organ culture dish in Brinster's BMOC-3 medium supplemented with penicillin and streptomycin. Epididymal sperm from each F1 male was used to inseminate oocytes from two females with a final sperm concentration of 3×10^4 sperm per dish. This concentration of sperm achieves slightly less than maximum fertilization in naive B6D2F1 mice, thus increasing the assay sensitivity in detecting changes in sperm fertilizing ability (unpublished data). Due to practical considerations, it was not possible to pool and distribute

oocytes from all females to inseminate a fixed number of oocytes per male, since oocytes were contained with a cumulus mass and oocyte collection was coordinated with sperm collection and CASA analysis for consistency among inseminations. Following insemination, oocytes were incubated at 37ºC under a humidified 5 % CO_2 environment. Following a 24 h incubation, 50 µl of 35 µM bisbenzimide stain (Sigma) was added to the petri dish. The oocytes were incubated with stain for at least 30 min before being examined using a Nikon Optophot fluorescent microscope equipped with a 100-W mercury bulb, 365/10 nm excitation filter, 400 nm dichromic mirror and 400 nm barrier filter. Oocytes were counted and scored as fertilized if the eggs were at the 2-cell stage or at the 1-cell stage containing two pronuclei and a second polar body. Oocytes were also evaluated for fragmentation and degeneration.

Statistical analysis

All data analysis was performed using SAS version 7 (SAS Inc., Cary, NC). For the analysis of F1 data, the litter was considered the experimental unit. F0 data and F1 litter mean data were analyzed for normality using the Shapiro-Wilk test. Evidence for non-normality was declared at the $p<0.01$ level of significance. Non-normal data was analyzed by nonparametric one-way ANOVA using the NPAR1WAY procedure of SAS. Comparisons between control and treated groups were made with the Kruskal-Wallis test. Normal data were analyzed using a one-way ANOVA as implemented in the MIXED procedure of SAS containing fixed effect of dose. When body and organ weights and anogenital distance of F1 mice were analyzed, litter size was included as a

covariate. Organ weights were analyzed as both absolute weight and as a percentage of body weight. AGD was analyzed as absolute length and as a ratio to the cube root of body weight (151). Comparisons between treatment groups were computed on least squares means. The effect of treatment on discrete data (fecundity, sperm IVF data) was analyzed using Generalized Estimating Equations as implemented in the GENMOD procedure of SAS. The level of significance was $\alpha < 0.05$. P values less 0.1 are also reported.

RESULTS

Analysis of lactational exposure to PCBs and chlorobiphenylol metabolites

The stomachs of 4 randomly selected F1 female mice in the 50 mg/kg dose group were dissected at 4 or 5 d of age and analyzed for PCBs, as well as di-, tri-, tetra-, and pentachlorobiphenylols (OH-PCBs) by HRGC/LRMS (Table 1-3). One stomach was also dissected from an F1 female in the corn oil control group to determine the level of background PCB and chlorobiphenylol contamination. Seventy individual PCB congeners and total PCBs were quantitated and compared to the congener and total PCB profile of Aroclor 1242 in order to estimate the level of lactational PCB exposure and to determine if the congener profile of PCBs was altered through maternal metabolism and lactational transfer. The 50-mg/kg dose group was selected in order to ensure detectable levels of PCBs and OH-PCBs in the stomach samples. Detection limits and quantitation of the 70 congeners was based on the height of individual peaks exceeding a 3:1 signal to noise threshold. Total PCBs and subtotals for each structural class were calculated from an integration of the chromatograph, rather than by summation of the 70 individual congeners that were examined (Table 1). The average total PCB content of the stomach samples from the 50-mg/kg dose group was 8659 ± 3312 ng/g. Assuming that the milk made up approximately 90% of the weight of the stomach sample and nursing pups consume an estimated 0.5 g of milk per day (0.25 g milk/g body weight), it is

Table 1. Congener profile of Aroclor 1242 and stomach samples from mouse offspring nursing from Aroclor 1242-treated mothers in the 50 mg/kg/2 day dose group.

IUPAC #[a]	Structure	Retention Time (min)	Aroclor 1242 (ng/g Aroclor)	50 mg/kg/2 day[b] (ng/g stomach)
	Monochlorobiphenyls			
1	2	16.594	1.3	ND (4)
3	4	17.882	0.56	ND (4)
Subtotal[c]			1.98	ND (4)
	Dichlorobiphenyls			
4/10	2,2'/2,6	18.511	3.1	ND (4)
8	2,4'	19.641	9.0	82 ± 3 (2)
15	4,4'	20.763	2.9	ND (4)
Subtotal			13.0	82 ± 3 (2)
	Trichlorobiphenyls			
16	2,2',3	21.161	7.7	ND (4)
18	2,2',5	20.712	13	ND (4)
19	2,2',6	20.153	0.95	ND (4)
22	2,3,4'	22.187	3.4	ND (4)
28	2,4,4'	21.799	16	1800 ± 658 (4)
33	2',3,4	22.015	7.1	ND (4)
37	3,4,4'	23.092	2.8	ND (4)
Subtotal			56	1800 ± 658 (4)
	Tetrachlorobiphenyls			
44	2,2',3,5'	23.013	4.4	235 ± 126 (4)
49	2,2',4,5'	22.663	3.9	355 ± 152 (4)
52	2,2',5,5'	22.564	4.4	748 ± 317 (4)

Table 1 (cont'd)

54	2,2',6,6'	21.479	ND	ND (4)
56	2,3,3',4	24.263	3.3	510 ± 216 (4)
64	2,3,4',6	23.283	4	109 ± 65 (4)
66	2,3',4,4'	23.916	4	1473 ± 717 (4)
70	2,3',4',5	23.835	4.6	157 ± 90 (3)
74	2,4,4',5	23.765	3.2	1103 ± 503(4)
77	3,3',4,4'	25.148	0.44	27 ± (1)
81	3,4,4',5	24.952	ND	ND (4)
Subtotal			35	4550 ± 1907 (4)
	Pentachlorobiphenyls			
87	2,2',3,4,5'	24.946	0.71	122 ± 47 (4)
95	2,2',3,5',6	23.933	0.94	70 ± 19 (3)
96	2,2',3,6,6'	23.388	ND	ND (4)
99	2,2',4,4',5	24.512	0.62	458 ± 202 (4)
101	2,2',4,5,5'	24.4	1.3	81 ± 27 (4)
104	2,2',4,6,6'	22.93	ND	ND (4)
105	2,3,3',4,4'	26.186	0.50	455 ± 159 (4)
110	2,3,3',4',6	25.145	0.99	45 ± 14 (4)
114	2,3,4,4',5	25.89	0.08	47 ± 3 (3)
118	2,3',4,4',5	25.657	0.84	668 ± 247 (4)
119	2,3',4,4',6	24.647	ND	ND (4)
123	2',3,4,4',5	25.616	ND	15 (1)
126	3,3',4,4',5	26.809	ND	ND (4)
Subtotal			5.3	1919 ± 721 (4)
	Hexachlorobiphenyls			
128/167	2,2',3,3',4,4'/2,3',4,4',5,5'	27.174	0.04	26 ± 7 (3)
138	2,2',3,4,4',5'	26.641	0.14	103 ± 29 (4)
149	2,2',3,4',5',6	25.628	0.10	ND (4)
151	2,2',3,5,5',6	25.395	0.03	ND (4)

Table 1 (cont'd)

153	2,2',4,4',5,5'	26.098	0.07	74 ± 21 (4)
155	2,2',4,4',6,6'	24.221	ND	ND (4)
156	2,3,3',4,4',5	27.615	0.01	15 ± 5 (3)
157	2,3,3',4,4',5	27.725	0.01	ND (4)
158	2,3,3',4,4',6	26.691	ND	ND (4)
168	2,3',4,4',5',6	26.156	0.05	ND (4)
169	3,3',4,4',5,5'	28.318	ND	ND (4)
Subtotal			0.51	208 ± 72 (4)
	Heptachlorobiphenyls			
170	2,2',3,3',4,4',5	28.483	0.01	ND (4)
171	2,2',3,3',4,4',6	27.607	0.01	ND (4)
174	2,2',3,3',4,5,6'	27.413	0.01	ND (4)
177	2,2',3,3',4',5,6	27.519	0.01	ND (4)
178	2,2',3,3',5,5',6	26.804	ND	ND (4)
179	2,2',3,3',5,6,6'	26.404	ND	ND (4)
180	2,2',3,4,4',5,5'	27.924	0.01	14.5 ± 1 (2)
183	2,2',3,4,4',5',6	27.061	0.01	ND (4)
187	2,2',3,4',5,6,6'	26.966	0.01	ND (4)
188	2,2',3,4',5,6,6'	25.987	ND	ND (4)
189	2,3,3',4,4',5,5'	29.003	ND	ND (4)
191	2,3,3',4,4',5',6	28.058	ND	ND (4)
Subtotal			0.05	14.5 ± 1 (2)
	Octachlorobiphenyls			
194	2,2',3,3',4,4',5,5'	29.643	ND	ND (4)
195	2,2',3,3',4,4',5,6	29.303	ND	ND (4)
199	2,2',3,3',4,5,5',6'	28.644	0.01	ND (4)
200	2,2',3,3',4,5,6,6'	28.18	ND	ND (4)
201	2,2',3,3',4,5',6,6'	27.737	ND	ND (4)
202	2,2',3,3',5,5',6,6'	27.589	ND	ND (4)

Table 1 (cont'd)

203	2,2',3,4,4',5,5',6	28.743	ND	ND (4)
205	2,3,3',4,4',5,5',6	29.752	ND	ND (4)
Subtotal			0.01	ND (4)
	Nonachlorobiphenyls			
206	2,2',3,3',4,4',5,5',6	29.278	ND	ND (4)
208	2,2',3,3',4,5,5',6,6'	30.328	ND	ND (4)
Subtotal			ND	ND (4)
	Decachlorobiphenyls			
209	2,2',3,3',4,4',5,5',6,6'	30.88	ND	ND (4)
Total				8659 ± 3312 (4)

[a] IUPAC number based on Ballschmiter et al. (1) and revised by Guitart et al. (2).
[b] Based on average of 4 stomach samples collected from 4 or 5 day old pups. Values in mean ± SD (detected in n samples).
[c] Total PCBs and subtotal concentrations are based on integration of the chromatograph and not on the summation of the individual congeners listed.
ND - non detectable. Detection limits and quantitation of the 70 congeners was based on the height of individual peaks exceeding a 3:1 signal to noise threshold.

estimated that the nursing pups were exposed to 0.24, 0.6, 1.2, and 2.4 mg/kg/d total PCBs in the 10, 25, 50, and 100 mg/kg dose groups, respectively. These estimates are based on linear extrapolations from the 50 mg/kg group. Indeed, analysis of total PCB concentration in one stomach sample from the 100 mg/kg dose group was approximately twice that of the 50 mg/kg sample (data not shown) and there were no detectable PCBs in the corn oil control stomach sample. The limit of detection for the corn oil control sample ranged from 3 to 20 ng/g.

There were very little di-CBs and no detectable mono-CBs in the stomach samples, which is similar to human breast milk (152). The detection limit for the stomach samples ranged from 5 to 20 ng/g for the mono-CBs and 10 to 50 ng/g for the di-CBs. The total amount of each PCB structural class was calculated as a percentage of the total PCBs in the sample based on the average congener concentrations in Table 1. The relative proportion of structural classes of PCBs within the stomach samples was slightly different than of the parent Aroclor mixture and of PCBs found in human breast milk (Table 2; (150)). For example, the predominant structural class of PCBs in the parent mixture was 50.9% tri-CBs, whereas the stomach sample consisted of 20.8% tri-CBs, which more closely resembles the percent of tri-CBs in human breast milk (14.6%) (152). The predominant structural class of PCBs in the stomach sample was tetra-CBs (55.1%), followed by penta (22.2%) and tri-CBs (20.7%). There was considerably less di-CBs in the stomach sample (0.9%) in comparison to the parent mixture (11.8%). Both the stomach samples

Table 2. Percent abundance of PCB congeners detected (based on ng PCB/g sample) within each structural class in Aroclor 1242 as a fraction of total PCBs in Aroclor 1242, stomach samples from mouse pups nursing on Aroclor 1242-treated mothers in the 50 mg/kg dose group, and human breast milk.

PCB Structural Class	Aroclor 1242	50 mg/kg[a]	Human Breast Milk[b]
Mono	1.8	ND	ND
Di	11.8	0.9	ND
Tri	50.9	20.8	14.6
Tetra	31.8	55.1	18.9
Penta	4.8	22.2	15.3
Hexa	<1.0	2.4	34.4
Hepta	<0.1	0.2	18.8
Octa	<0.1	ND	3.0
Nona	ND	ND	0.2
Deca	ND	ND	0.1

[a] Based on average of 4 stomach samples collected from 4 or 5 day old pups.
[b] From Safe *et al.* (3).
ND - non detectable

and parent mixture had relatively low to no detectable levels of hexa- to deca-CBs. In contrast, the human breast milk sample consisted of primarily hexa-CBs (34 %) and roughly equivalent amounts of tri-, tetra-, penta-, and hepta-CBs (14.6 to 18.9%). Human breast milk also had no detectable mono- or di-CBs (152).

The relative abundance of OH-PCBs parallels the relative abundance of the non-hydroxylated congeners, however the total amount of OH-PCBs represents only about 5% of the total PCBs + OH-PCBs in the 50 mg/kg sample (Table 3). The predominate OH-PCBs were tri- and tetrachlorobiphenylols, which represented at least 74% of the total OH-PCBs detected. Penta-CBs represented about 20% of the total OH-PCBs in the stomach samples. The identities of the OH-PCBs were not determined, however, there were on average 33.8 ± 10 different OH-PCBs species per sample based on the number of unique peaks identified in the chromatograms. The majority of the peaks were found to be tri- (14 ± 3.9) and tetra-OH-CBs (16.8 ± 5.2 peaks).

Reproductive performance of F0 generation

Exposure of F0 female C57BL6 mice to Aroclor 1242 prior to mating and through gestation and lactation did not significantly affect body weight gain or liver weight (Table 4). Although body weights in the 25 mg/kg dose group were significantly different ($p<0.05$) from control mice prior to dosing, the difference was not apparent by the mating period. There was a decrease ($p=0.0523$) in average body weight in the 50 mg/kg dose group on PND 21, however weight gain was not affected. There was approximately a 50% increase ($p<0.05$) in

Table 3. Chlorobiphenylols in stomach samples from pups nursing from Aroclor 1242-treated mothers in the 50 mg/kg/2 day dose group.

OH-PCB	50 mg/kg/2 day[a]	Average number of peaks
Total Dichlorobiphenylol	37 ± 34 (2) [7.1]	2.0 ± 1.4 (2)
Total Trichlorobiphenylol	199 ± 118 (4) [38 %]	14 ± 3.9 (4)
Total Tetrachlorobiphenylol	197 ± 109 (4) [37.7 %]	16.8 ± 5.2 (4)
Total Pentachlorobiphenylol	45 ± 17 (3) [17.2 %]	2.7 ± 0.6(3)
Total Di to Pentachlorobiphenylol	448 ± 237 (4) [100]	33.8 ± 10 (4)

Values in ng OH-PCB/g stomach ± SD (detected in n samples) [% of total].
[a] Based on average of 4 stomach samples collected from 4 or 5 day old pups.

Table 4. Body and organ weights and reproductive performance of F0 female mice gavaged with Aroclor 1242 through gestation and lactation[a].

	Dose of Aroclor 1242 (mg/kg/2 days)				
	0	10	25	50	100
Body weight, g					
Pre-dosing	20.74 ± 0.41 (10)	19.76 ± 0.40 (8)*	22.02 ± 0.09 (7)**	20.59 ± 0.39 (8)	20.93 ± 0.33 (7)
Pre-mating	21.73 ± 0.23 (10)	21.25 ± 0.36 (8)	21.74 ± 0.20 (7)	21.64 ± 0.39 (8)	21.76 ± 0.27 (7)
PND 21	30.93 ± 0.56 (9)	29.68 ± 0.77 (8)	30.56 ± 0.62 (7)	28.96 ± 0.93 (7)*	30.27 ± 0.33 (3)
Weight gain[b]	10.17 ± 0.93 (9)	9.90 ± 1.01 (8)	8.42 ± 0.64 (7)	8.27 ± 0.82 (7)	9.36 ± 0.58 (3)
Liver weight, g	2.22 ± 0.12 (9)	2.16 ± 0.11 (8)	2.33 ± 0.13 (7)	2.17 ± 0.08 (7)	2.35 ± 0.04 (3)
Liver:body weight ratio*100	7.16 ± 0.35 (9)	7.27 ± 0.24 (9)	7.64 ± 0.35 (7)	7.49 ± 0.21 (7)	7.76 ± 0.22 (3)
Thymus weight, mg	30.83 ± 4.70 (9)	34.23 ± 5.88 (8)	46.89 ± 4.11 (7)**	31.69 ± 5.46 (7)	29.43 ± 6.35 (3)
Thymus:body weight ratio*100	0.10 ± 0.01 (9)	0.12 ± 0.02 (8)	0.15 ± 0.01 (7)**	0.11 ± 0.02 (7)	0.10 ± 0.02 (3)
Fecundity					
# of breeding pairs	16	9	10	11	11
# with live births	10	8	7	8	5
% Fecundity	62.5	88.9	70.0	72.7	45.5[e]
Days to parturition[c]	21.6 ± 1.8 (10)	21.0 ± 1.4 (8)	20.7 ± 1.1 (7)	20.9 ± 1.1 (7)	21.2 ± 1.1 (5)
Litter size on PND 0	7.6 ± 0.3 (10)	7.5 ± 0.6 (8)	7.9 ± 0.4 (7)	8.1 ± 0.6 (8)	6.0 ± 1.1 (5)*
Litter weight on PND 1, g	12.17 ± 0.48 (10)	12.17 ± 1.66 (8)	11.29 ± 0.49 (7)	13.24 ± 0.94 (7)	10.45 ± 1.27 (4)
Average pup weight on PND 1, g	1.60 ± 0.02 (10)	1.62 ± 0.08 (8)	1.45 ± 0.08 (7)*	1.54 ± 0.05 (7)	1.54 ± 0.10 (4)
Sex ratio, % male	0.44 ± 0.04 (10)	0.51 ± 0.06 (8)	0.55 ± 0.09 (7)	0.44 ± 0.06 (7)	0.71 ± 0.04 (4)***
Table 4 (cont'd)					
Survival[d]	90 ± 10 (10)	100 ± 0 (8)	91.2 ± 3.6 (7)	84.5 ± 12.2 (8)	80.0 ± 20.0 (5)

Table 4 (cont'd)

[a] F0 female C57BL6 mice were dosed orally every other day for two weeks prior to being paired with an untreated male DBA/2 and were continually dosed every other day until postnatal day (PND) 21.
[b] Difference between initial body weight and body weight on postnatal day 21.
[c] Average number of days between pairing of female with male and parturition.
[d] (Number of pups alive on PND 21/number of pups alive on PND 0) x 100, %
[e] Significantly less ($P < 0.0347$) than 10 mg/kg dose group.
All values are means ± SEM of (n) mice. Different from control group at the following level of significance. *$0.1 < p < 0.05$; **$0.05 < p < 0.01$; *** $p < 0.01$.

thymus weight and percent thymus weight in the 25 mg/kg maternal dose group ($p<0.05$) on PND 21. There were no other significant changes in thymus weight in the PCB-treated groups.

Only 5 of 11 (45.5 %) females in the 100 mg/kg dose group gave birth to live young. Due to the low fecundity in the control group (10/16, 62.5 %), there was no significant difference in fecundity when compared to controls ($p>0.1$). However, when fecundity in the 100 mg/kg dose group was compared to the 10 mg/kg dose group, the decrease in fecundity was significant ($p<0.05$). Four mothers in the 100 mg/kg dose group showed no evidence of conception, while one mother appeared pregnant based on weight gain on day 16 after the initial pairing with a male, there was no parturition. However, uteri from these mice were not examined for implantation scars so it is unclear if conception had occurred. One mother in the 100 mg/kg group gave birth to stillborn pups. Two of three pups in one litter died on PND 1, while the third pup died on PND 6. The remaining 4 litters survived to PND 21; however, one mother died on PND 20 from unknown causes and the pups were subsequently removed from the study. Due to the small number of litters remaining in the 100 mg/kg dose group, no conclusions regarding treatment-related effects in F1 males could be determined with confidence, although the results are presented.

There was no significant difference between treatment groups in the number of days to parturition from the initiation of pairing with a male ($p>0.1$). There was a decrease in litter size (6.0 ± 1.1 vs 7.6 ± 0.3, $p<0.01$) in the 100 mg/kg dose group, although no difference in litter weight was detected ($p>0.1$).

There was a small decrease in average pup weight ($p=0.077$). There were no significant differences in pup survival, however there was a smaller proportion of females in the 100 mg/kg dose group when compared to controls ($p<0.01$). Due to the small sample size and the unknown sex of the pups that died on PND1, the true sex ratio is unknown. There was no postweaning F1 mortality throughout the study.

Developmental effects

There were no significant effects on body weight, AGD, or AGD:cube root body weight ratio on PND 21; however there was a slight increase ($p=0.092$) in average AGD:cube root body weight ratio in the 25 mg/kg dose group (Table 5). There was no effects on body, liver, thymus, or testis weight at 16 or 45 weeks of age ($p>0.1$, Table 5).

Sperm count and motion analysis

Cauda epididymal sperm from F1 males were analyzed on a computer-assisted sperm analysis system for sperm count and various motion parameter (Table 6). Comparison of the sperm counts among 16 week old F1 mice indicated that mice from the 25 mg/kg maternal dose group had a 36% greater average sperm count that was close to statistical significance ($p=0.064$). Sperm count was not affected in other treatment groups ($p> 0.1$). There was also a significant increase in sperm velocity ($p<0.05$) and linearity ($p<0.01$) in the 25 mg/kg dose group. All other motion parameters were unaffected by PCB exposure except for an increase ($p=0.098$) in sperm linearity in the 50 mg/kg dose group. By 45 weeks of age, average sperm count of F1 mice in the 25

Table 5. Body and organ weights and anogenital distance (AGD) of male mice exposed to Aroclor 1242 through gestation and lactation.

Male	Dose of Aroclor 1242 (mg/kg/ 2 days) 0	10	25	50	100[a]
AGD, mm	6.56 ± 0.29 (9)	6.31 ± 0.44 (8)	6.99 ± 0.23 (7)	6.33 ± 0.35 (6)	7.67 ± 0.53 (3)
AGD:cube root body weight ratio, mm/g$^{1/3}$	3.29 ± 0.11 (9)	3.19 ± 0.17 (8)	3.52 ± 0.10 (7)*	3.22 ± 0.14 (6)	3.66 ± 0.18 (3)
Body weight, g					
PND 21	7.94 ± 0.39 (9)	7.66 ± 0.49 (8)	7.81 ± 0.20 (7)	7.55 ± 0.30 (6)	9.20 ± 0.69 (3)
16 weeks	27.24 ± 0.59 (8)	28.24 ± 0.80 (8)	28.19 ± 1.05 (7)	27.52 ± 0.49 (7)	28.72 ± 1.21 (3)
45 weeks	40.60 ± 1.18 (8)	40.82 ± 1.35 (8)	42.65 ± 0.32 (6)	39.70 ± 1.75 (6)	43.26 ± 2.64 (3)
Liver weight, g					
16 weeks	1.50 ± 0.08 (8)	1.48 ± 0.04 (8)	1.48 ± 0.07 (7)	1.42 ± 0.06 (7)	1.47 ± 0.19 (3)
45 weeks	1.82 ± 0.05 (8)	1.92 ± 0.07 (8)	1.85 ± 0.02 (6)	1.88 ± 0.08 (6)	1.98 ± 0.19 (3)
Liver:body weight ratio*100					
16 weeks	5.49 ± 0.24 (8)	5.24 ± 0.14 (8)	5.43 ± 0.29 (7)	5.14 ± 0.13 (7)*	5.08 ± 0.49 (3)
45 weeks	4.51 ± 0.12 (8)	4.72 ± 0.11 (8)	4.33 ± 0.06 (6)	4.79 ± 0.10 (6)	4.57 ± 0.38 (3)
Thymus weight, mg					
16 weeks	37.2 ± 2.9 (9)	36.8 ± 3.8 (8)	41.1 ± 1.1 (7)	36.8 ± 2.3 (7)	19.6 ± 9.1 (3)**
45 weeks	43.1 ± 3.7 (8)	42.0 ± 4.2 (8)	48.1 ± 2.0 (6)	46.7 ± 4.4 (6)	50.5 ± 9.3 (3)
Thymus:body weight ratio*100					
16 weeks	0.14 ± 0.01 (9)	0.13 ± 0.01 (8)	0.15 ± 0.004 (7)	0.13 ± 0.1 (7)	0.07 ± 0.03 (3)**
45 weeks	0.11 ± 0.01 (8)	0.10 ± 0.01 (6)	0.11 ± 0.004 (6)	0.12 ± 0.01 (6)	0.12 ± 0.02 (3)
Testes weight, mg					
16 weeks	204.9 ± 8.9 (8)	204.3 ± 7.3 (8)	213.4 ± 12.4 (7)	220.1 ± 5.6 (7)	216.2 ± 6.4 (3)

Table 5 (cont'd)

45 weeks	222.1 ± 6.5 (8)	221.1 ± 8.8 (8)	207.6 ± 13.4 (6)	220.3 ± 10.0 (6)	251.3 ± 10.4 (3)
Testes:body weight ratio*100					
16 weeks	0.75 ± 0.02 (8)	0.73 ± 0.03 (8)	0.78 ± 0.03 (7)	0.80 ± 0.02 (7)	0.75 ± 0.02 (3)
45 weeks	0.55 ± 0.02 (8)	0.54 ± 0.02 (8)	0.49 ± 0.03 (6)	0.57 ± 0.03 (6)	0.58 ± 0.01 (3)

[a] Due to the small number of litters remaining in the 100 mg/kg dose group, no conclusions regarding treatment-related effects in F1 males could be determined with certainty, although the results are presented.

All values are means ± SEM of (n) litters. Different from control group at the following level of significance. *$0.1 < p < 0.05$; **$0.05 < p < 0.01$; *** $p < 0.01$.

Table 6. Epididymal sperm count and sperm motion of 16 and 45 week old F1 male mice exposed to Aroclor 1242 through gestation and lactation.

	Dose of Aroclor 1242 (mg/kg/2 days)				
16 week old	0	10	25	50	100[a]
Sperm count, x10^6/ml	36.56 ± 4.67 (8)	32.27 ± 3.92 (8)	49.74 ± 4.44 (7)*	41.86 ± 8.82 (7)	38.99 ± 5.35 (3)
Motility, %[b]	69.3 ± 4.5 (8)	63.7 ± 3.6 (8)	71.1 ± 2.5 (7)	70.7 ± 4.3 (7)	69.5 ± 3.6 (3)
Velocity, µm/s	131.19 ± 5.39 (8)	127.25 ± 3.91 (8)	145.95 ± 2.85 (7)**	130.72 ± 5.47 (7)	133.01 ± 5.99 (3)
Linearity	4.84 ± 0.13 (8)	4.84 ± 0.17 (8)	5.56 ± 0.17 (8)***	5.17 ± 0.24 (7)*	5.14 ± 0.08 (3)
ALH displacement	4.96 ± 0.43 (8)	4.98 ± 0.16 (8)	5.34 ± 0.4 (7)	4.99 ± 0.42 (7)	5.05 ± 0.38 (3)
Tail cross frequency, Hz	11.84 ± 0.60 (8)	10.86 ± 0.46 (8)	11.65 ± 0.79 (7)	11.78 ± 0.52 (7)	12.26 ± 0.71 (3)
45 week old					
Sperm count, x10^6/ml	39.58 ± 4.42 (8)	44.42 ± 1.92 (8)	31.63 ± 5.66 (6)	41.84 ± 5.66 (6)	44.31 ± 5.37 (3)
Motility, %	65.3 ± 3.4 (8)	67.7 ± 2.8 (8)	63.6 ± 4.1 (8)	64.1 ± 2.9 (6)	62.3 ± 10.1 (3)
Velocity, µm/s	134.46 ± 2.94 (8)	141.99 ± 3.87 (8)	128.62 ± 3.03 (6)	135.56 ± 2.80 (6)	144.83 ± 5.16 (3)
Linearity	5.01 ± 0.17 (8)	5.20 ± 0.20 (8)	4.77 ± 0.22 (6)	4.92 ± 0.17 (6)	5.42 ± 0.58 (3)
ALH displacement	4.78 ± 0.28 (8)	5.55 ± 0.27 (8)*	4.77 ± 0.27 (6)	5.85 ± 0.44 (6)*	5.61 ± 0.83 (2)
Tail cross frequency, Hz	11.58 ± 0.51 (8)	12.17 ± 0.50 (8)	11.37 ± 0.73 (6)	12.17 ± 0.74 (6)	14.53 ± 1.30 (2)**

Table 6 (cont'd)

[a] Due to the small number of litters remaining in the 100 mg/kg dose group, no conclusions regarding treatment-related effects in F1 males could be determined with certainty, although the results are presented.
All values are means ± SEM of (n) litters. Different from control group at the following level of significance. *$0.1 < p < 0.05$; **$0.05 < p < 0.01$; *** $p < 0.01$.
[b] Sperm motion parameters were measured after 30 min. in capacitation medium.

mg/kg dose group was similar to that of control mice ($p>0.1$). Again, the other dose groups were not affected. There were no significant effects on sperm motion parameters except for an increase in average ALH displacement in the 10 ($p=0.067$) and 50 mg/kg ($p=0.090$) dose groups.

***In vitro* fertilizing ability**

Oocytes from untreated female B6D2F1 mice were inseminated *in vitro* with cauda epididymal sperm (30,000 sperm cells/mL) from F1 males and evaluated for fertilization 24 h later. Sperm from PCB-exposed F1 mice fertilized significantly ($p<0.001$) fewer eggs than sperm from control F1 mice at both 16 and 45 weeks of age, with the exception of the 50 mg/kg dose group at 16 weeks of age (Table 7). At 16 weeks of age, sperm from the 10, 25, and 100 mg/kg maternal dose groups fertilized 10%, 29%, and 15% fewer oocytes. At 45 weeks of age, sperm from the 10, 25, 50, and 100 mg/kg maternal dose group fertilized 14%, 28%, 17%, and 27% fewer oocytes than control sperm, respectively. There was a significant ($p<0.05$) decrease in the percent of fragmented oocytes in the 50 mg/kg dose group at 16 weeks of age and in the 25 and 50 mg/kg dose group at 45 weeks of age, however, the magnitude of effects were small. There was a significant increase ($p<0.05$) in the percent of 1 cell fertilized eggs in the 10 mg/kg dose group at 16 weeks of age. There was no difference in the percent of 1 cell fertilized eggs at 45 weeks of age (Table 7)

Table 7. *In vitro* fertilizing ability of cauda epididymal sperm from 16 and 45 week old male mice exposed to Aroclor 1242 through gestation and lactation [a].

	Dose of Aroclor 1242 (mg/kg/alternate day)				
16 weeks old	0	10	25	50	100[b]
Percent fertilized	83.2 (583/701)	75.0 (392/523)***	58.8 (332/566)***	84.3 (702/833)	70.6 (324/459)***
Percent fragmented	4.1 (29/701)	3.3 (17/523)	3.2 (18/566)	1.9 (16/833)**	1.7 (8/459)**
Percent 1 cell fertilized	0.6 (4/701)	1.9 (10/523)**	0.7 (4/566)	1.0 (8/883)	0.4 (2/459)
45 week old					
Percent fertilized	76.7 (440/574)	65.6 (548/835)***	55.3 (271/490)***	63.6 (419/659)***	55.8 (202/362)***
Percent fragmented	2.4 (14/574)	1.8 (15/835)	0.6 (3/490)**	0.8 (5/659)**	3.6 (13/362)
Percent 1 cell fertilized	0.4 (2/574)	0.5 (4/835)	0.2 (1/490)	0.5 (2/659)	0.6 (2/362)

[a] Eggs were considered fertilized if they were at the two-cell stage or the one-cell stage with two pronuclei. Degenerate eggs were not included in the analysis of percent eggs fertilized.

[b] Due to the small number of litters remaining in the 100 mg/kg dose group, no conclusions regarding treatment-related effects in F1 males could be determined with certainty, although the results are presented.

Different from control group at the following level of significance. *$0.1 < p < 0.05$; **$0.05 < p < 0.01$; *** $p < 0.01$.

DISCUSSION

Due to the ability of Aroclor 1242 to disrupt normal signaling of both estrogen and thyroid hormones *in vivo* (reviewed in (140), we hypothesized that Aroclor 1242 may impact spermatogenesis and reproductive development in males exposed through gestation and lactation. Decreases in adult testis size and sperm production following prenatal and neonatal exposure to estrogenic chemicals have been well documented (82,153). In contrast, increases in adult testis size and sperm production in males exposed neonatally to goitrogenic compounds and Aroclor 1242 and 1254 have also been reported (147,154). Based on previous studies (144,146,147), it was expected that male offspring exposed to Aroclor 1242 through gestation and lactation would exhibit an increase in adult testis size and sperm production, but reduced fertility. However, differences in species, timing of exposure, dose, and the age when males are examined may influence the observed effects.

In this study, treatment of pregnant mice with doses of Aroclor 1242 up to 50 mg/kg/2-d did not seriously impact maternal health or reproduction. Body weight gain and organ weights of treated F0 mice were not significantly different from control F0 mice, indicating PCB-treatment had no general systemic toxicity. The significant increase in thymus weight in F0 mice in the 25 mg/kg dose group may have been due to the weak estrogenic activity of Aroclor 1242, since estrogen is known to play an important role in controlling thymus size (155). However, the increase was not linearly related to dose. There was an apparent

17% decrease in average fecundity in the 100 mg/kg dose group, although the results were not statistically significant due to the abnormally low fecundity in the control group (Table 4). However, when the 100 mg/kg dose group was compared to the 10 mg/kg dose group, there was a 45% decrease in fecundity that was significant. The low fecundity in the controls was due to 6 of 16 females not conceiving, whereas 3 litters in the 100 mg/kg group were either stillborn, experienced early F1 death, or late F0 death. Since it is unknown whether the failure to give birth to live young was due to fetal abortion and/or resorption, it cannot be concluded that Aroclor 1242 caused reproductive failure at the doses used in this study. However, treatment of mature female rats with 150 mg/kg/day Aroclor 1242, but not 75 mg/kg/day, has been shown to decrease serum progesterone and abolish reproductive success (156). The ability of PCBs, such as Aroclor 1242 and selected non-coplanar *ortho*-substituted congeners, to stimulate oscillatory contractions of pregnant rat uterine muscle could contribute to the disruption of pregnancy, in addition to effects on progesterone levels (157). In any event, effects on the offspring in the current study, at least in the 10, 25, and 50 mg/kg dose groups, are not likely to be due to compromised maternal health but rather are likely to be mediated through direct gestational and lactational exposure.

Gestational exposure was not measured in this study, however, placental transfer of PCBs is well documented. Lactational exposure was estimated based on the PCB concentration determined from stomach samples obtained from nursing pups. An estimate of the level of lactational exposure to PCBs was 0.2,

0.6, 1.2, and 2.4 mg/kg/day total PCBs in the 10, 25, 50, and 100 mg/kg dose groups, respectively. This level of exposure approaches the maximum FDA recommended level for food and breast milk (4 ppm). In addition to the chlorobiphenylols in the stomach samples, nursing pups were exposed to a mixture of PCBs containing a higher proportion of penta-, hexa-, hepta-CBs and a lower proportion of mono- and di-CBs in comparison to the parent Aroclor 1242 mixture. This exposure profile is more similar to human milk samples than to the parent mixture (Table 2 and 3). This difference is likely due to the amenable metabolism and excretion of the lower chlorinated biphenyls and retention of the more persistent highly chlorinated biphenyls by the mothers. This is evident from the higher proportion of trichlorobiphenylols found in the stomach samples (Table 3). A large number of PCBs found in the stomach samples were *ortho*-substituted PCBs containing at least one *para*-substituted chlorine (Table 6). This structure has been found to be optimal for estrogen receptor binding and estrogenic activity *in vitro* (158,159). *Ortho*-substituted congeners with lateral substitution have also been shown to bind to transthyretin, a thyroxine and retinol binding and transport protein (160). *Para*-hydroxylation of *ortho*-substituted PCBs increases the estrogen receptor binding affinity (159), but decreases the affinity for transthyretin (160). The extent of hydroxylation of the tri- and tetra-CBs is likely to be large, since there were on average 34 individual peaks in the chromatogram. Although the identity of the hydroxylated species was not determined, *para*-hydroxylation of PCBs is a preferred reaction in rat (161).

In this study, gestational and lactational exposure of mice to Aroclor 1242 increased average sperm count in 16 week old mice, although the magnitude of the effect was smaller than observed in rats (147), not linearly related to dose, and only close to being statistically significant (p=0.064) (Table 6). In contrast to the effects in rats, testis weight was not significantly increased at 16 weeks of age (Table 5). There is no evidence in this study that androgen status of F1 mice was affected since AGD was not decreased following PCB-exposure (Table 5). The observed effect of Aroclor 1242 on rat sperm production is thought to be due to a thyroid-dependent mechanism, since goitrogen (PTU)-induced hypothyroidism in the neonatal rat has also been shown to increase adult testis weight and daily sperm production (162). Indeed, thyroxine replacement was found to eliminate or decrease the increased testis weight and sperm production in Aroclor 1242- and 1254-treated rats (147). Hypothyroidism may be accounted for, in part, by increased thyroxine conjugation and excretion by UDP-glucuronosyltransferases (UDPGT) following enzyme induction by PCBs and Aroclor 1254 (140). Consistent with this hypothesis is the observation that *in utero* exposure of mice to the coplanar congener 3,4,3',4'-tetrachlorobiphenyl (PCB77) increased testis size (148). However, acute treatment of some noncoplanar congeners and Aroclor 1242 can decrease serum thyroxine concentration (140). One hypothesis is that the PCBs and/or their hydroxylated metabolites compete with thyroxine for binding to transthyretin, which results in an increase in the free fraction of thyroxine, thus increasing conjugation and excretion (163). There is no evidence in this study that thyroid status in PCB-

exposed mice was affected since testes weight and sperm count was not significantly increased. Mice also appear to be less sensitive to chemically-induced thyroid alterations when compared to rats (164). These discrepancies may also be due to the timing and route of exposure, dose, or species differences.

Neonatal exposure to Aroclor 1254 has previously been observed to decrease sperm fertilizing ability in 45 week old mice (150). In contrast to the trends observed in the 25 mg/kg maternal dose group at 16 weeks of age, 45 week old mice in this dose group had average testis weight and average sperm counts similar to the controls. Changes in spermatogenesis and testis size in hypothyroid rats increase to a maximum at 160 days of age (162). It is unknown whether these changes persist to middle age or older. Most studies examining the effects of developmental exposure to endocrine disrupting chemicals on male fertility have focused on younger adults (< 45 weeks old), while the persistence and manifestation of effects at later stages of life have been relatively ignored (84,165).

Neonatal exposure of rats to Aroclor 1242 does not affect fertility in breeding studies (147). However, one study has shown adverse affects on fertility in 18 week old rats following neonatal exposure to Aroclor 1254 (143). These effects were not accompanied by a decrease in epididymal sperm count or changes in sperm morphology or motility, but rather a decline in the ability of sperm to fertilize eggs (144,146). Therefore, a more sensitive assay to detect changes in the ability of sperm to fertilize eggs from Aroclor 1242 exposed males

at 16 and 45 weeks of age was used. At 16 weeks of age, epididymal sperm from all but the 50 mg/kg maternal dose group showed a significant decline in the number of oocytes fertilized *in vitro*, with the 25 mg/kg dose group being affected most dramatically. At 45 weeks of age, epididymal sperm fertilizing ability was significantly decreased in all dose groups and the 25 mg/kg dose group was again most affected. These changes were not related to apparent changes in sperm motion. The fertilized ova were able to progress to the 2 cell stage greater than 98% of the time and there was no increase in oocyte fragmentation, but rather a small but significant decrease in the 25 and 50 mg/kg dose groups (Table 7). Adverse effects on human sperm have also been observed in young men exposed prenatally to PCBs and polychlorinated dibenzofurans (i.e. Yu-Cheng exposure), including sperm motility, velocity, beat cross frequency and hamster oocyte penetration ability (142). However, sperm count and semen volume were not significantly affected. In the current study, testis size and epididymal sperm count were also not predictive of effects on *in vitro* fertility. Other reproductive parameters, such as hormone levels and testicular sperm count, have also been shown not to be predictive of fertility (84). The advantage of the IVF assay is the increased sensitivity in detecting adverse effects on sperm fertilizing ability, since dramatic reductions (> 80%) in sperm production are usually required prior to observing effects on fertility in breeding studies (166). *In vitro* fertility studies, however, do not provide information on whether sperm function is affected as a result of testicular, sperm or epididymal malfunction. Considering the normal testes size, epididymal sperm count and

sperm motility among treatment groups, the malfunction(s) responsible for the reduced fertility *in vitro* may be more subtle than gross structural lesions or germ cell differentiation. These malfunctions may involve biochemical, structural, or functional changes within the spermatazoa as a result of alterations in the expression of genes involved in chromosomal packaging, acrosome function, or other processes involved in fertilization. These changes could have occurred during spermiogenesis in the gonad or sperm maturation in the epididymis.

In this study, gestational and lactational exposure to Aroclor 1242 does not increase testes size and epididymal sperm count in 16 or 45 week old mice. Testis size, epididymal sperm count and motility were not predictive of the decline in fertility *in vitro*, which occured in both young adult and middle age mice. Furthermore, these effects occurred in male offspring that were exposed *in utero* and through lactation at levels approaching the maximum FDA recommended level for food and breast milk (4 ppm). Based on these and other studies, it appears that adverse effects on fertility due to developmental exposure to PCBs can occur in both humans and rodents in the absence of significant changes in testis size or sperm production. This indicates that changes at the molecular or biochemical level may have occurred during testicular development and/or sperm maturation to negatively impact sperm fertilizing ability. Gene and protein expression profiling technologies may be useful for identifying these changes in the somatic and germ cells of the male gonad and reproductive tract.

CHAPTER 5

GESTATIONAL AND LACTATIONAL EXPOSURE OF MALE MICE TO DIETHYLSTILBESTROL: LONG TERM EFFECTS ON TESTICULAR DEVELOPMENT AND SPERM FERTILIZING ABILITY *IN VITRO*[3]

ABSTRACT

The objective of the study was to determine the long-term effects of gestational and lactational exposure to diethylstilbestrol (DES) on testicular growth and histology, number of Sertoli cells, epididymal sperm count and motility, and sperm fertilizing ability *in vitro* in B6D2F1 mice. Pregnant females were gavaged daily with 0, 0.1, 1, or 10 µg DES in corn oil per kg of maternal body weight from gestational day 12 to postnatal day (PND) 21. Male neonates were monitored for body weight and anogenital distance (AGD) and weaned on PND21. The testes from male offspring were examined on PND21, 105 and 315 for changes in wet weight, histopathology and number of Sertoli cells. Seminal vesicle weight was also measured on PND105 and 315. Epididymal sperm count, sperm motion parameters and sperm fertilizing ability *in vitro* were measured on PND105 and 315. There were no significant effects on AGD or seminal vesicle weight at any time point, and no incidence of gross reproductive

[3] Submitted for publication in Fielden M.R., Halgren R.G., Staub C., Johnson L., Chou K., Zacharewski T.R. (2002) Gestational and lactational exposure of male mice to diethylstilbestrol: I. long-term effects on testicular development and sperm fertilizing ability *in vitro*.

tract abnormalities, except for one animal in the high dose group (unilateral cryptorchidism). In addition, no significant changes in testes weight were observed and histological examination of the testes revealed no treatment-related effects. However, stereological analysis of the testes indicated a significant decrease in the number of Sertoli cells per testis in the high dose group, which persisted from PND21 to PND315 ($p<0.01$). Sperm count was also decreased in the high dose group, but the decrease was only significant on PND315 ($p<0.05$). The number and percent of motile sperm, and sperm velocity, linearity and amplitude of lateral head displacement were unaffected. However, *in vitro* fertilizing ability of epididymal sperm was significantly decreased in the high dose group on both PND105 ($p<0.001$) and PND315 ($p<0.05$). On PND105, there was also a large increase in the percent of fertilized eggs that did not proceed to the two-cell stage. Interestingly, fertilizing ability was significantly increased ($p<0.001$) on PND315 in the 0.1 µg/kg group. These results demonstrate that developmental exposure to DES can cause long-term, potentially irreversible, alterations in sperm fertilizing ability *in vitro* in the absence of histological changes in the testis, reproductive tract abnormalities or alterations in sperm motion parameters.

INTRODUCTION

It was suggested as early as 1974 that the quality of human semen has been declining over the past 50 to 60 years (1). In 1992, Carlsen et al. (2) published a meta-analysis of 61 studies that reported a significant decrease in sperm concentration and seminal volume between 1940 and 1990. However, this analysis has been questioned by several authors for a number of methodological reasons (3,4). Furthermore, significant geographical differences in sperm count have since been detected between different regions, thus further confounding interpretation of these studies (9-11). Nonetheless, the incidence of testicular cancer has increased 2 to 4 % per year during the same time period (12). Male reproductive tract malformations, such as cryptorchidism and hypospadia, also appear to be slightly increased (14).

A common environmental factor has been suggested as a possible contributor to the reported decline in male reproductive health. Many of the effects are similar to those observed in human males exposed *in utero* to the synthetic estrogen diethylstilbestrol (DES). Sharpe and Skakkebaek subsequently hypothesized that *in utero* exposure to environmental and dietary estrogens may compromise male reproductive health (15). This hypothesis was supported not only by clinical effects in human males, but also by reports of compromised reproductive fitness of wildlife populations in proximity to areas polluted with environmental contaminants having estrogenic activity (i.e. xenoestrogens) (167,168).

An essential role for the estrogen receptor (ER)α and estrogen synthesis in male reproductive tract development, spermatogenesis and fertility has been confirmed following the generation of ERα and aromatase knockout mice (37,44). However, the role of endogenous estrogen in the development and function of the male reproductive tract is still unclear. Furthermore, the assertion that synthetic estrogenic chemicals can adversely affect human reproductive health is still controversial. This is largely due to the relatively weak potency and low level of exposure to xenoestrogens in comparison to dietary derived phytoestrogens, and the relatively high dose of xenoestrogens necessary to cause adverse effects in laboratory animals (49-51).

DES is a potent synthetic non-steroidal estrogen that was widely used in the late 1940's to the late 1960's to prevent miscarriages for high risk pregnancies. It has also been used to suppress lactation, control menopausal symptoms, treat breast and prostate cancer, and as an abortificient (54). Tragically, its reproductive, teratogenic and carcinogenic effects subsequently became evident in the offspring of pregnant mothers prescribed DES. Studies have since concluded that prenatal exposure to DES causes and vaginal and cervical clear-cell adenocarcinoma in female offspring following otherwise normal pubertal development (55,56). Putative effects in human males include testicular cancer, hypospadias, cryptorchidism, lower sperm concentration, and impaired fertility (reviewed in (169,170). Although administration of DES to pregnant women was halted in 1971, DES has since become the prototypical estrogenic

chemical for studying the endogenous role of estrogen in male reproductive tract development and the pathotoxicology of exogenous estrogen exposure (73,171).

Although the effects of prenatal exposure to DES on male rodents have been well established, the effects on spermatogenesis are uncertain. Prenatal exposure of male mice to DES (≥100 µg/kg maternal body weight) causes sterility and a number of reproductive tract abnormalities, including enlarged and cystic Müllerian remnants, inhibition of gubernaculum development and cryptorchidism, sperm granulomas, hypotrophic testes and epididymides, epididymal cysts of embryonic female origin, and tumors of the rete testis and interstitial cells (74-80,172-174). These lesions likely contribute directly to sterility since adverse effects on fertility at lower doses (<100 µg/kg) or doses that do not cause reproductive tract abnormalities have not been described, and therefore warrant further investigation. Gestational and lactational exposure of rats to 50 µg/l DES in drinking water (~8.6 µg/kg/day) caused a small but significant decrease in testis and epididymis weight and testicular and epididymal sperm counts (81). Sharpe et al have also observed small but significant decreases in testes weight and daily sperm production in rats exposed through gestation and lactation to 100 µg/l DES in drinking water (82). However, these results were not confirmed in repeat studies (83). In any event, it is unclear whether gestational and lactational exposure to DES, or other xenoestrogens, can adversely affect male fertility at non-teratogenic doses since changes in sperm production or organ weights do not adequately predict sperm function and quality (84).

The purpose of this study was to determine if gestational and lactational exposure to non-teratogenic doses of DES causes long-term effects on testes development, sperm count and motility, and *in vitro* fertilizing ability. Doses of 0.1, 1 and 10 µg/kg/day were used in order to minimize or avoid reproductive tract abnormalities that may confound the interpretation of effects on sperm quality. Sperm fertilizing ability was assessed using an *in vitro* fertilization (IVF) assay to avoid confounding treatment effects on sexual behavior, sperm count, or development of external genitalia and accessory glands. Male offspring were examined at early and mid stages of life to determine the persistence of the treatment-related effects. In addition to the effects on F1 males described here, Chapter 6 describes the treatment-related effects on testicular gene expression using cDNA microarrays and real-time PCR.

MATERIALS AND METHODS

Animals

Mice were obtained from Charles River Laboratories (Portage, MI) and housed in polycarbonate cages with cellulose fiber chips (Aspen Chip Laboratory Bedding, Northeastern Products, Warrensberg, NY) as bedding and maintained in a humidity (30-40%) and temperature (23ºC) controlled room on a 12 h light-dark cycle. All animals were given free access to deionized water in glass bottles with rubber stoppers and AIN-76A rodent feed *ad libitum* (Research Diets, New Brunswick, NJ). This diet is a casein-based open-formula purified diet with non-detectable levels of the estrogenic isoflavones genistein, diadzein or glycitein (unpublished data and (175,176).

F0 treatment

Eleven week old virgin C57BL/6 female mice (F0) were housed two per cage upon arrival and acclimatized for at least three days. Each pair of females was housed with 7- to 8-week old DBA/2 proven breeder male within a week of arrival. Offspring from this cross, designated B6D2F1, were used since a large number of eggs can be obtained from superovulated B6D2F1 mice, a high level of fertilization can be consistently and reproducibly obtained in control animals (85,148). Following evidence of pregnancy, dams were separated from the males, housed individually and sequentially assigned to one of four treatment groups. Because of the low fecundity of dams in the high dose group, additional pregnant mice were allocated in order to generate a sufficient number of viable

litters per group (see Results). Due to the limited number of male offspring that can be examined for sperm analysis and IVF at any one day, breeding was staggered into four cohorts (i.e. replicates) to accommodate sperm analysis for all the male offspring. Each cohort included time-matched control animals.

F0 mice were treated by daily gavage with 0.1 ml of corn oil (Sigma, St. Louis, MO) for a nominal dose of 0, 0.1, 1, and 10 µg DES per kg of maternal body weight (09:00 – 12:00) from GD12 to PND20. There was a one-day interruption of treatment on the day of parturition (PND0). The dose of test chemical was adjusted daily to body weight for each dam before dosing. Offspring were weaned on PND21 when F0 mice were euthanized. The F0 treatment and F1 necropsy schedule is shown in Figure 1.

F1 development

Litter size and litter weight were recorded on PND0 and PND1, respectively. F1 body weight was measured on PND7 and PND21. Anogenital distance (AGD; the length of the perineum from the base of the genital tubercle to the center of the anus when the skin was naturally extended without stretching) was measured on PND7 and 21. Measurements were recorded to the nearest 0.1 mm and obtained with a dissecting scope equipped with an ocular micrometer (Nikon, Melville, NY). AGD was measured by the same individual to increase precision and to control for operator variation. F1 mice were weaned on PND21, housed with same sex littermates, and fed AIN-76A *ad libitum* until necropsy.

F1 necropsy

On PND21, one F1 male per litter was euthanized for necropsy. In the instances where the number of F1 males in a litter was low, the male(s) were instead withheld for necropsy on PND105 and 315 when male offspring were also euthanized for assessment of sperm quality. The remaining F1 males from each litter were randomly selected for sperm quality assessment on either PND105 or 315 (Figure 1). At necropsy, both testes were excised, trimmed of adhering connective tissue and weighed wet. Both seminal vesicles with coagulating glands were excised and weighed wet on PND105 and 315. At all three time points, one testis was fixed for histology and the other testis was stored immediately for subsequent RNA extraction (Chapter 6).

Testicular histopathology

One testis per animal was fixed in 15 to 20 volumes of Bouin's fixative (Sigma) for at least 24 h and cleared in three successive 1 h washes in 70 % ethanol. Wet tissue was stored in 70 % ethanol at room temperature before being shipped to Experimental Pathology Laboratories (Durham, NC) for analysis. Each testis was embedded in paraffin and three 5 micron cross sections from the cranial, median, and caudal part of the testes were stained with hematoxylin and eosin. All three sections were mounted on the same slide and evaluated microscopically by pathologists blind to the treatment group.

Stereological analysis of Sertoli cells

Paraffin blocks were deparaffinized and further fixed in 1 % osmium in sodium cacodylate buffer and embedded in epon. Tissues were sectioned at 0.5

μm, stained with toluidine blue, and used for stereological determination of the volume density (percentage) of Sertoli cell nuclei (177). The average diameter was used to calculate a rough estimate of the volume of a single nucleus, assuming the nucleus to be a sphere. Since Sertoli cell nuclei are not spherical, a correction factor (0.633) was used to obtain a corrected final volume for an individual nucleus (178). The corrected final volume of a single nucleus was the product of the rough estimate times 0.663. The number of Sertoli cells per gram of parenchyma was calculated when the product of the volume density of Sertoli cell nuclei, parenchymal volume per gram (0.95), and the approximated histological correction factor for section thickness and nuclear diameter (179) was divided by the corrected volume of a single Sertoli cell nucleus. The number of Sertoli cells per testis was calculated by multiplying the number of Sertoli cells per gram of parenchyma by the weight of the testis.

Epididymal sperm count and motion analysis

Cauda epididymal sperm were collected from F1 males on PND105 and 315 by excising both epididymides and piercing with a 25 gauge needle in an organ culture dish (Becton Dickinson, Franklin Lakes, NJ) containing 1 ml of BMOC-3 medium (Gibco/BRL, Grand Island, NY), a capacitation supporting medium (180). Sperm suspensions were incubated at 37ºC in a humidified 5 % CO_2 air environment for 30 min before sperm concentration and motion analyses, and 60 min before insemination (see below). Sperm suspensions (20 μl) were placed on a 20 μm counting chamber and analyzed using a CellSoft computer-assisted digital image analysis system (CASA) Series 4000 (CRYO Resources

Ltd., Montgomery, NY). A minimum of 100 cells were analyzed to determine average sperm count, motility, number of motile sperm, velocity, linearity, amplitude of lateral head (ALH) displacement and beat/cross frequency for each animal. Sperm count represents the number of sperm in 1 ml of media. Motility is expressed as the percentage of sperm that move faster than 20 μm/s. Number of motile sperm was the product of sperm count and motility. Velocity is defined as the average distance (μm) traveled by motile sperm in 1 s. Linearity is the ratio of the straight to actual distance traveled (x10), averaged over all sperm. ALH displacement is a measure of the lateral movement of the sperm head from the curval mean of its track. The beat/cross frequency (Hz) is the numbers of beats (or crosses) of the sperm across its curval mean per second. All measurements for each sperm collection were performed in duplicate and averaged.

***In vitro* fertilization assay**

The *in vitro* fertilizing ability of sperm was assessed on PND105 and 315 by inseminating oocytes collected from non-treated, 3 to 4 week-old, B6D2F1 female mice. Female mice were superovulated with 10 IU pregnant mare's serum gonadotropin (Sigma) followed 48 h later with 10 IU human chorionic gonadotropin (Sigma). Fourteen to 17 h later the oocytes from each female were collected from the proximal oviducts. Oocytes from each mouse were incubated in a 1 ml organ culture dish containing 0.9 ml of Brinster's BMOC-3 medium. Epididymal sperm from each F1 male was used to inseminate oocytes from two females, each in a separate dish. Sperm were diluted in BMOC-3 medium and

0.1 ml of diluted sperm was added to 0.9 ml of medium containing the oocytes to achieve a final sperm concentration of 3 x 10^4 sperm per ml per dish. This concentration of sperm achieves slightly less than maximum fertilization in naive B6D2F1 mice, thus increasing the assay sensitivity for detecting positive and negative changes in sperm fertilizing ability. It was not possible to pool, randomize and equally distribute oocytes from all females since oocytes were contained within a cumulus mass and oocyte collection was coordinated with sperm collection and CASA analysis for consistency among inseminations. Following insemination, oocytes were incubated at 37ºC under a humidified 5% CO_2 air environment. Following a 24 h incubation, 50 µl of 35 µM bisbenzimide stain (Sigma) was added to the dish. The oocytes were incubated with stain for at least 30 min before being examined using a Nikon Optophot fluorescent microscope equipped with a 100-W mercury bulb, 365/10 nm excitation filter, 400 nm dichromic mirror and 400 nm barrier filter. Oocytes were counted and scored as fertilized if the eggs were at the 2-cell stage or at the 1-cell stage containing two pronuclei and a second polar body. Oocytes were also evaluated for fragmentation and other signs of degeneration. Since it was not always possible to distinguish a one-cell from a two-cell fragmented egg, they were not included in the total egg count for calculating percent fertilization. Fertility data for the replicate dishes were averaged.

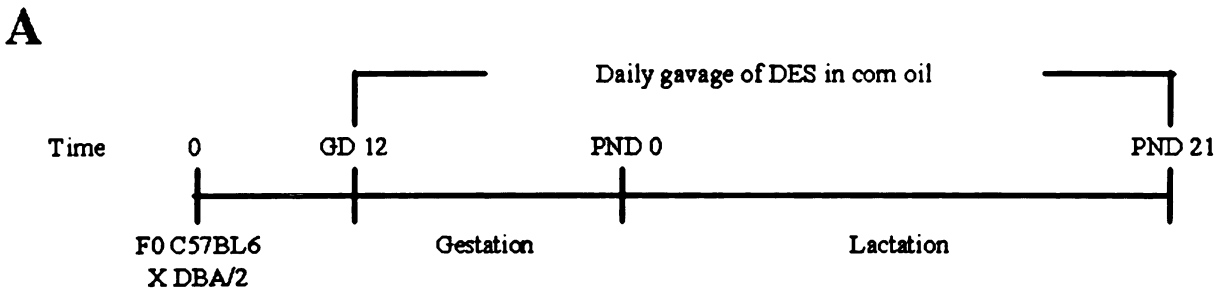
Statistical Analysis

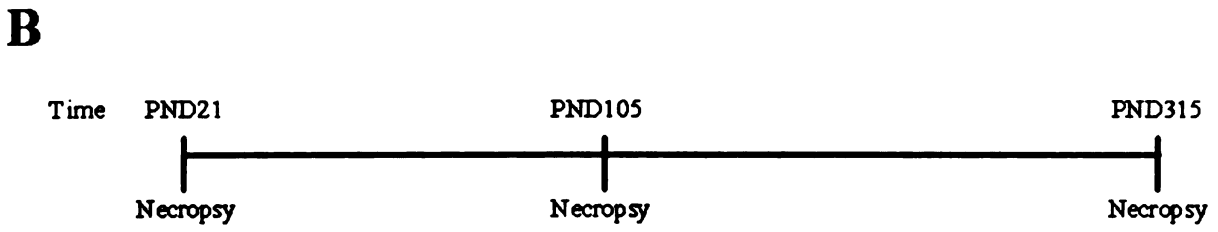
All data analysis was performed using SAS version 8.0 (SAS Inc, Cary, NC). To estimate experimental error, the litter was considered the experimental

unit. There was no significant ($p > 0.1$) replicate effect, therefore, data from all cohorts were pooled for analyses. All data were tested for normality by the Shapiro-Wilk test. Evidence for non-normality was declared at the 5% level of significance. When significant, the data was log transformed and re-tested. Data that were not normally distributed were analyzed by non-parametric one-way analysis of variance (ANOVA) using the NPAR1WAY procedure of SAS. Comparisons between control and treated groups were made with the Kruskal-Wallis test. Data passing the normality test were analyzed with a repeated measures ANOVA using the MIXED procedure of SAS. Using an analysis of covariance, litter size was found to account for a significant source of the variation ($p<0.05$). Therefore, for the analysis of body weight, the model included dose, time, and dose x time interaction as fixed effects and litter size as a covariate. Likewise, for the analysis of AGD and organ weights, body weight was included in the model as a covariate since body weight was found to account for a significant source of the variation ($p<0.05$) (181). For comparison, AGD was also analyzed as a ratio of AGD to the cube root of body weight (151), while organ weights were analyzed as a ratio of organ weight to body weight. Sperm count, sperm motion parameters and Sertoli cell morphometry were analyzed using dose, time, and dose x time interactions as fixed effects. Due to the unbalanced nature of the ANOVA, and to adjust group means for the covariate, comparisons between control and treated groups were performed on LS-means and adjusted for multiple comparisons by Dunnett's method. Arithmetic means, standard errors and n values for litters are reported in the results. The effect of

treatment on discrete data (i.e. proportion of eggs fertilized) was analyzed by logistical regression using a binomial distribution as implemented in the GENMOD procedure of SAS. The level of significance was set at $p<0.05$.

Figure 1. Treatment schedule for F0 mice and necropsy schedule for F1 male offspring. A) F0 C57BL6 mice were fed AIN-76A rodent diet and mated with a proven breeder male DBA/2. Pregnant mice were treated by daily gavage with 0.1 ml of corn oil for a nominal dose of 0, 0.1, 1, and 10 µg DES per kg of maternal body weight from gestational day (GD) 12 to postnatal day (PND) 21. B) Male B6D2F1 offspring were weaned on PND21 and housed with same-sex littermates. F1 males were necropsied on either PND21 (3 weeks of age), PND105 (15 weeks of age), or PND315 (45 weeks of age). Testes weight, histology and Sertoli cell stereology were examined at each time point. Epididymal sperm count, motion analysis and *in vitro* fertilizing ability were assessed on PND105 and PND315.


A
Daily gavage of DES in corn oil
Time
0
GD 12
PND 0
PND 21
F0 C57BL6
X DBA/2
Gestation
Lactation


B
Time
PND21
PND105
PND315
Necropsy
Necropsy
Necropsy

RESULTS

Reproductive performance of F0 generation

There was a significant decrease in F0 body weight on PND20 in the 10 µg/kg group, however, their body weight was slightly less than controls before treatment and as a result there was no difference in weight gain between pre-mating and PND20 (Table 1). The number of pregnant dams giving birth to live young (gestation index) was significantly decreased ($p<0.05$) in the 10 µg/kg group when compared to controls (Table 1). Of the 19 pregnant females in the 10 µg/kg group, only 11 (58%) gave birth to live young. Five of the pregnant females in the 10 µg/kg dose group gave birth to moribund pups, while three did not give birth at all and were euthanized. Two (of 16) pregnant females in the control group did not give birth and were also euthanized. Three (of 13) females in the 0.1 µg/kg group and two (of 14) females in the 1 µg/kg group gave birth to moribund pups. The pregnant females that did not give birth were verified by necropsy to be carrying fetuses. The live pups showed no differences in viability or weaning index, a measure of offspring survival to PND4 or PND21, respectively (Table 1). There was a significant ($p<0.01$) decrease in litter size in the 10 µg/kg group, which resulted in a significant decrease ($p<0.05$) in total litter weight (Table 1). The average pup weight, however, was not significantly affected by DES treatment. The percentage of males in the control group was an expected 50 %. The percentage of males in the 1 µg/kg group was slightly

Table 1. Reproductive performance of F0 females gavaged with diethylstilbestrol through gestational and lactation.

	Maternal dose of diethylstilbestrol (μg/kg/day)			
	0	0.1	1	10
Body weight				
Pre-mating	19.72 ± 0.45 (14)	19.82 ± 0.65 (13)	19.18 ± 0.40 (12)	18.89 ± 0.43 (11)
PND1	27.06 ± 0.35 (14)	26.70 ± 0.47 (13)	26.76 ± 0.25 (12)	25.86 ± 0.39 (11)
PND7	29.34 ± 0.45 (14)	28.08 ± 0.27 (13)	28.63 ± 0.42 (12)	27.98 ± 0.39 (11)
PND14	29.90 ± 0.44 (14)	29.02 ± 0.27 (13)	29.93 ± 0.58 (12)	29.32 ± 0.36 (11)
PND20	30.34 ± 0.47 (14)	28.76 ± 0.65 (13)	29.04 ± 0.35 (12)	28.27 ± 0.49 (11)*
Weight gain[a]	10.61 ± 0.57 (14)	8.94 ± 0.86 (13)	9.86 ± 0.48 (12)	9.38 ± 0.54 (11)
Number of pregnant mice	16	13	14	19
Number of viable litters	14	10	12	11
Gestation index[b]	87.5	76.9	85.7	57.9 *
Viability index[c]	98.2 ± 1.3 (14)	93.4 ± 3.0 (10)	95.5 ± 2.6 (12)	90.9 ± 9.1 (11)
Weaning index[d]	98.1 ± 1.3 (14)	91.4 ± 3.9 (10)	95.5 ± 2.6 (12)	90.9 ± 9.1 (11)
Litter size (PND 0)	9.0 ± 0.4 (14)	8.9 ± 0.6 (10)	8.0 ± 0.8 (12)	6.8 ± 0.5 (11)**
Litter weight (PND 1, g)	12.39 ± 0.45 (14)	12.40 ± 0.84 (10)	11.33 ± 0.82 (12)	9.67 ± 0.68 (11)*
Average pup weight (PND 1, g)	0.73 ± 0.02 (14)	0.73 ± 0.04 (10)	0.70 ± 0.04 (12)	0.71 ± 0.02 (11)
Table 1 (cont'd)				
Percent males	50.0 ± 3.5 (14)	46.2 ± 5.6 (10)	58.8 ± 2.8 (12)*	34.1 ± 7.5 (10)*

Table 1 (cont'd)

All values are means ± SE of (n) F0 mice.
* Significantly different from control group; * $p < 0.05$; ** $p < 0.01$.
a Difference between pre-mating body weight and final body weight on PND 20
b (Number of pregnant females giving birth to live young/number of pregnant females) x 100, %
c (Number of pups alive on PND 4/number of pups alive on PND 0) x 100, %
d (Number of pups alive on PND 21/number of pups alive on PND 0) x 100, %

greater than controls ($p<0.05$), while there was a large and significant decrease (32%; $p<0.05$) in the percentage of males in the 10 µg/kg group (Table 1).

F1 Development

Doses of DES were selected in order to minimize or avoid reproductive tract abnormalities yet fall within the range of human exposures. There was only one incident of unilateral cryptorchidism observed (out of 116 DES exposed offspring) in the 10 µg/kg group on PND315. Both testes and epididymides from this animal were hypotrophic and no sperm could be obtained. The retained testis was firmly attached to the seminal vesicle. Otherwise, there were no gross reproductive tract abnormalities observed in litter mates or any other male offspring in the control or DES-exposed groups. Although epididymis weight was not recorded, abnormally small epididymides were not observed in any animals. Treatment did not have a significant effect on body weight throughout the period of study (Table 2).

No significant treatment-related changes in AGD were observed on regardless of how AGD was corrected for body weight (Table 2). On PND21, testes weight was decreased approximately 30% in the 10 µg/kg group relative to the control group; however, the results were not significant. However, when testes weight was adjusted for body weight (i.e. mg testis/g body weight), the decrease became was significant ($p<0.05$). Since the relationship between body weight and testes weight was not linear (data not shown), a change in testes weight on PND21 can not be concluded (181). On PND105 and 315, testes weight was decreased approximately 11 % in the 10 µg/kg group

Table 2. Body and organ weights and anogenital distance of F1 male mice exposed to diethylstilbestrol through gestation and lactation[a].

	Maternal dose of diethylstilbestrol (μg/kg/day)			
	0	0.1	1	10
Body weight (g)				
PND 7	3.88 ± 0.09 (14)	3.99 ± 0.17 (10)	4.14 ± 0.21 (12)	4.04 ± 0.18 (9)
PND 21	8.06 ± 0.27 (14)	8.76 ± 0.42 (10)	8.51 ± 0.50 (12)	7.57 ± 0.33 (10)
PND 105	25.73 ± 0.91 (13)	25.32 ± 0.66 (9)	25.58 ± 1.18 (11)	24.45 ± 1.38 (6)
PND 315	42.71 ± 0.92 (13)	45.90 ± 1.36 (9)	42.66 ± 1.78 (11)	40.64 ± 1.62 (7)
AGD (mm)[a]				
PND 7	2.79 ± 0.06 (14)	2.81 ± 0.06 (10)	2.81 ± 0.05 (12)	2.82 ± 0.04 (9)
PND 21	6.84 ± 0.29 (14)	7.20 ± 0.33 (10)	7.12 ± 0.42 (12)	6.31 ± 0.16 (10)
Adjusted AGD ($mm/g^{1/3}$)[b]				
PND 7	1.77 ± 0.03 (14)	1.78 ± 0.04 (10)	1.76 ± 0.02 (12)	1.78 ± 0.04 (9)
PND 21	3.41 ± 0.12 (14)	3.49 ± 0.11 (10)	3.48 ± 0.14 (12)	3.22 ± 0.05 (10)
Testes weight (mg)[a]				
PND 21	45.19 ± 2.03 (13)	48.09 ± 3.67 (9)	45.59 ± 4.47 (11)	31.73 ± 4.60 (6)
PND 105	173.97 ± 5.52 (13)	167.48 ± 5.60 (9)	167.59 ± 6.22 (11)	155.16 ± 8.34 (6)[c]
PND 315	190.08 ± 4.43 (13)	194.96 ± 7.16 (9)	182.90 ± 5.46 (11)	179.12 ± 17.28 (6)
Adjusted testes weight (mg/g)[d]				
PND 21	0.55 ± 01 (13)	0.55 ± 0.02 (9)	0.55 ± 0.02 (11)	0.42 ± 0.04 (6)*
PND 105	0.69 ± 0.03 (13)	0.66 ± 0.02 (11)	0.66 ± 0.02 (11)	0.61 ± 0.03 (6)
PND 315	0.45 ± 0.02 (13)	0.43 ± 0.02 (9)	0.44 ± 0.02 (11)	0.44 ± 0.04 (6)

Table 2 (cont'd)

Seminal vesicle weight (mg)[a]				
PND 105	268.15 ± 15.90 (13)	254.73 ± 9.50 (9)	262.75 ± 17.71 (11)	270.46 ± 20.12 (6)
PND 315	529.21 ± 16.46 (13)	580.65 ± 33.13 (9)	526.34 ± 41.22 (11)	469.33 ± 17.51 (6)
Adjusted seminal vesicle weight (mg/g)[c]				
PND 105	1.04 ± 0.04 (13)	1.03 ± 0.05 (9)	1.02 ± 0.03 (11)	1.06 ± 0.06 (6)
PND 315	1.24 ± 0.03 (13)	1.26 ± 0.05 (9)	1.22 ± 0.05 (11)	1.16 ± 0.03 (6)

All values are litter means ± SE (n).
* Significantly different from control group; * $p < 0.05$.
a Body weight was included as a covariate in the ANOVA, as described in Materials and Methods.
b Adjusted for the cube root of body weight at time of AGD measurement
c p=0.0603
d Adjusted for body weight at time of necropsy

relative to the control group, although the differences were not significant. No significant changes in seminal vesicle weight were observed on PND105 or 315 (Table 2).

Histopathology

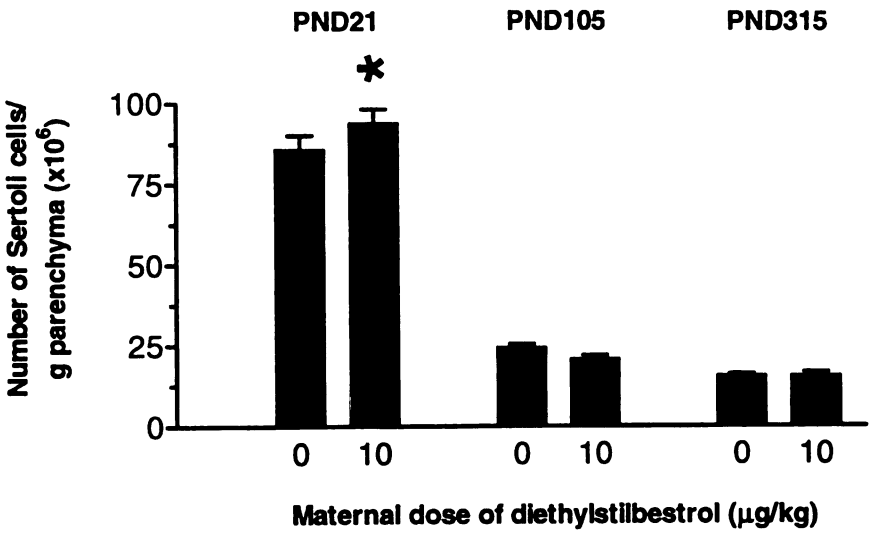
Testes from all dose groups on PND21 were examined microscopically, while only samples from the control and 10 µg/kg group on PND105 and 315 were examined microscopically. No treatment related histological changes could be identified in any sections when assessed blind to the treatment. As expected, the sections from PND21 indicated that few to no elongated spermatids or spermatozoa could be identified, while on PND105 and 315, all testes were producing spermatozoa.

Stereological analysis of Sertoli cells

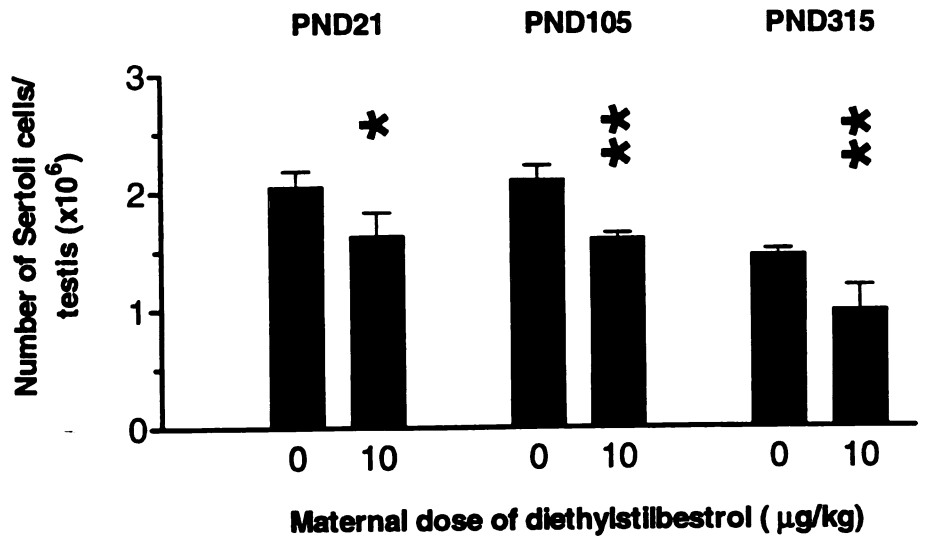
Stereological analysis of the Sertoli cells was conducted only on the testes from the control group and the 10 µg/kg group due to the observed differences in testes weight and sperm count at this dose (see below). On PND21 there was a significant decrease ($p<0.05$) in number of Sertoli cells per gram of parenchyma in the 10 µg/kg group, but no differences on PND105 or 315 (Figure 2A). When standardized to testes weight the number of Sertoli cells per testis was significantly decreased 25, 17 and 32 % relative to controls on PND21, 105 and 315, respectively (Figure 2B). Based on the decrease in the number of Sertoli cells per testis, the non-significant, but modest decreases in testes weight in the 10 µg/kg group reported in Table 2 are likely treatment-related.

Figure 2. Stereological analysis of Sertoli cell number in F1 males exposed to DES through gestation and lactation. A) Number of Sertoli cells per gram of parenchyma on PND21 (control n=7; DES n=5), PND105 (control n=8; DES n=6) and PND315 (control n=13; DES n=5) in male offspring. B) Number of Sertoli cells per testis on PND21, 105 and 315 in male offspring. Asterisks indicate significantly different from respective time-matched control group: * $p < 0.05$, ** $p < 0.01$.

A


PND21
PND105
PND315
*
100
75
50
25
0
Number of Sertoli cells/ g parenchyma (x10^6)
0
10
0
10
0
10
Maternal dose of diethylstilbestrol (µg/kg)

B


PND21
PND105
PND315
*
**
**
3
2
1
0
Number of Sertoli cells/ testis (x10^6)
0
10
0
10
0
10
Maternal dose of diethylstilbestrol (µg/kg)

Epididymal sperm count and motion analysis

Cauda epididymal sperm from F1 males were analyzed on a computer-assisted sperm analysis system for sperm count, motile count (the number of sperm moving faster than 20 μm/s) and other motion parameters (Table 3). Sperm count and the number of motile sperm was decreased approximately 19% and 28%, respectively, in the 10 μg/kg group on PND105, although the results were not significant. Similarly, there was a 26% and 27% decrease in sperm count and the number of motile sperm in the 10 μg/kg group on PND315, with the decrease in sperm count being significant ($p<0.05$). There was a dose-dependent increase in beat-cross frequency that was significant ($p<0.05$) in the 1 and 10 μg/kg group on PND315. Sperm motility, velocity, linearity and ALH displacement were not significantly affected by DES, however, these parameters were all lower on average in the 10 μg/kg group relative to controls on PND105 and 315 (Table 3).

***In vitro* Fertilizing Ability**

Fertilizing ability *in vitro* was measured in duplicate dishes for each animal. The average difference between duplicate measurements in control animals was less than 12%, indicating that *in vitro* fertility measurements were reproducible. Sperm fertilizing ability on PND105 in control animals was approximately 60 %. On PND315, fertilizing ability in the control mice was approximately 44% (Table 4).

On PND105, there was a modest but significant decrease in sperm fertilizing ability in the 1 μg/kg group ($p<0.05$) and the 10 μg/kg group ($p<0.001$).

Table 3. Epididymal sperm quality of F1 male mice exposed to diethylstilbestrol through gestation and lactation.

	Maternal dose of diethylstilbestrol (µg/kg/day)			
	0	0.1	1	10
Sperm count (x10^6/ml)				
PND 105	28.00 ± 2.26 (13)	28.98 ± 2.16 (8)	27.77 ± 2.96 (11)	22.79 ± 2.94 (6)
PND 315	48.66 ± 2.36 (13)	44.54 ± 3.04 (9)	41.89 ± 3.77 (11)	35.92 ± 8.39 (6)*
Motility (% > 20 µm/s)				
PND 105	61.70 ± 2.55 (13)	58.32 ± 4.40 (8)	56.86 ± 2.90 (11)	55.55 ± 2.53 (6)
PND 315	74.22 ± 1.69 (13)	74.58 ± 2.20 (9)	71.26 ± 2.37 (11)	70.71 ± 3.98 (6)
Motile count (x10^6/ml)[a]				
PND 105	18.00 ± 2.02 (13)	17.37 ± 2.25 (8)	16.60 ± 2.49 (11)	13.01 ± 1.97 (6)
PND 315	36.42 ± 2.44 (13)	33.61 ± 2.95 (9)	30.70 ± 3.56 (11)	26.73 ± 7.80 (6)
Velocity (µm/s)				
PND 105	126.53 ± 2.20 (13)	128.36 ± 2.67 (8)	125.72 ± 3.48 (11)	122.56 ± 2.73 (6)
PND 315	142.65 ± 1.78 (13)	138.24 ± 2.37 (9)	138.93 ± 1.98 (11)	136.00 ± 3.52 (6)
Linearity				
PND 105	4.98 ± 0.09 (13)	4.75 ± 0.16 (8)	4.94 ± 0.14 (11)	4.85 ± 0.17 (6)
PND 315	5.46 ± 0.11 (13)	5.09 ± 0.10 (9)	5.28 ± 0.08 (11)	5.41 ± 0.22 (6)

Table 3 (cont'd)

ALH displacement (μm)				
PND 105	5.16 ± 0.25 (13)	4.76 ± 0.37 (8)	4.78 ± 0.20 (11)	4.33 ± 0.37 (6)
PND 315	5.31 ± 0.21 (13)	5.02 ± 0.18 (9)	5.03 ± 0.30 (11)	4.36 ± 0.37 (6)
Tail cross frequency (Hz)				
PND 105	14.09 ± 1.83 (13)	12.51 ± 0.91 (8)	12.17 ± 0.32 (11)	12.19 ± 0.44 (6)
PND 315	11.21 ± 0.55 (13)	12.40 ± 0.64 (9)	12.88 ± 0.38 (11)*	13.17 ± 0.39 (6)*

All values are litter means ± SE (n)
* Significantly different from control group; $p < 0.05$.
a Number of sperm with velocity greater than 20 μm/s (i.e. product of sperm count and motility)

Table 4. In *vitro* fertilizing ability of epididymal sperm from F1 male mice exposed to diethylstilbestrol through gestation and lactation.

	Maternal dose of diethylstilbestrol (µg/kg/day)			
	0	0.1	1	10
Number of litters (number of animals)				
PND 105	13 (24)	8 (12)	11 (20)	5 (9)
PND 315	13 (21)	9 (13)	11 (20)	6 (7)
% Fertilizing ability (fertilized/total eggs)				
PND 105	59.6 (1129/1896)	62.7 (676/1079)	55.5 (973/1752)*	50.9 (266/523)***
PND 315	43.5 (411/944)	67.8 (387/871)***	43.9 (382/871)	36.4 (168/462)*
% Fragmented eggs (fragmented/total eggs)				
PND 105	2.3 (44/1896)	1.4 (15/1079)	2.1 (36/1752)	2.7 (14/523)
PND 315	3.3 (31/944)	0.9 (5/571)**	4.1 (36/871)	1.1 (5/462)**
% One-cell fertilized (fertilized/total eggs)[a]				
PND 105	2.0 (37/1896)	0.8 (9/1079)*	1.3 (23/1752)	6.3 (33/523)***
PND 315	0.6 (6/944)	0 (0/571)*	0.2 (2/871)	0 (0/462)*

* Significantly different from control group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.
a Percent of eggs that did not proceed to the two-cell stage

There was no significant change in the number of fragmented eggs, but there was a large and significant increase ($p<0.001$) in the number of fertilized eggs in the 10 µg/kg group that did not proceed to the two-cell stage (Table 4). There was also a slight but significant decrease ($p<0.05$) in the number of one-cell fertilized eggs in the 1 µg/kg group. The significant decrease in sperm fertilizing ability in the 10 µg/kg group persisted to PND315 ($p<0.05$). However, there was an unexpected large increase in fertilizing ability in the 0.1 µg/kg group that was significant ($p<0.001$). There was a significant decrease in the percent of one-cell fertilized eggs in the 0.1 and 10 µg/kg group, however this was due to the absence of any one-cell fertilized eggs observed in these groups. There was also a slight, but significant decrease ($p< 0.01$) in the number of fragmented eggs in the 0.1 and 10 µg/kg group.

DISCUSSION

This study has demonstrated a long-term decrease in the number of Sertoli cells, epididymal sperm count and sperm fertilizing ability following gestational and lactational exposure to 10 µg/kg/day of DES. The effects on testes weight are equivocal, although the decrease in the number of Sertoli cells supports a true decrease in testes weight. Furthermore, the effects on sperm fertilizing ability and Sertoli cell number occurred in the absence of reproductive tract abnormalities, histopathology, or effects on sperm motility. These results underscore the importance of assessing functional endpoints on sperm quality, as changes in testes weight, histology or sperm motion and morphology do not adequately predict fertility *in vivo* or sperm fertilizing ability *in vitro* (84,85). Previous studies have shown that teratogenic effects can be induced by maternal subcutaneous exposure to 50 µg DES/kg/day or greater during mid-gestation (74,75,172). The sterility observed in offspring exposed to such high doses of DES is probably secondary to cryptorchidism, hypoplastic testes or epididymides, and/or Müllerian remnants, which would severely disrupt spermatogenesis. In the present study, there was only one incidence of unilateral cryptorchidism, and no evidence of hypoplastic testes or epididymides, or Müllerian remnants. Spermatogenesis was histologically normal and reproductive development of the offspring was also grossly normal. Therefore, the long-term effects of DES exposure on sperm quality are likely due to effects on the development and

maturation of the sperm via direct action on the germ cells, the supporting somatic cells of the testes, and/or the epididymides.

Maternal effects by themselves are unlikely to account for the effects on testicular development or sperm quality since treatment-related effects appeared to be limited to the offspring. The decrease in the number of live offspring born, in addition to the decrease in litter size and litter weight in the 10 µg/kg group, suggest that DES was causing intrauterine death. The decrease in the percent of male offspring in the 10 µg/kg group (Table 1) suggests that the male might be particularly sensitive to the fetotoxicity of DES, although the decrease in sex ratio may be a chance occurrence, as previously observed in other studies (182). Similar effects on reproductive performance of pregnant rodents have been previously observed at comparable or higher doses of DES (75,183-185). The reduced number of live offspring was not due to effects on ovulation, implantation loss or inhibition of implantation, since treatment began well after implantation, which occurs on GD 5 to 6 in mice. The fetal abortion and/or death observed in the 10 µg/kg group may occur through alterations in the maternal hypothalamic-pituitary-gonadal axis leading to impaired function of the ovaries or uterus, or by direct action on the endometrium or placenta leading to abnormal parturition (186). This is supported by the abnormal placental development of DES-treated pregnant mice (187).

It is expected that most of the effects caused by combined gestational and lactational exposure to DES are mediated primarily, if not exclusively, during gestational exposure. Although maternal estrogen exposure is well known to

suppress lactation, the doses required are much higher than those used in the present study (188), where no treatment-related effects on offspring body weight were observed (Table 2). Transplacental movement of DES has been well documented (189), but lactational movement of DES in rodents has not been investigated to our knowledge. Based on the measurement of lactational transfer of steroidal compounds (190,191), it can be conservatively estimated that approximately 0.1 percent of the administered dose of DES is transferred to each nursing pup. This would result in a daily dose of approximately 0.00025 µg/pup/day in the high dose group (dose x body weight x 0.1% / No. of pups), which is far less than the lowest neonatal dose of DES (0.01 µg/pup/day) reported to causes measurable effects on spermatocytes and plasma follicle-stimulating hormone (FSH) levels in prepubertal rats (192). Furthermore, exposure of lactating rats to extremely high doses of DES (10,000 µg/kg) does not cause urogenital abnormalities in male or female offspring (75). However, the lowest observable effect level for neonatal exposure to DES is unknown, so the contribution of lactational DES exposure cannot be completely discounted. Furthermore, treatment-related alterations in milk composition may also impact neonatal development (193).

This is the first study to demonstrate that adverse effects on sperm quality following gestational and lactational exposure to DES can persist to at least PND315 in mice. By contrast, DES-induced effects on testes weight and sperm production at non-teratogenic doses are limited to observations on male offspring less than 137 days old (79,81,82). Latent effects on spermatogenesis following

neonatal DES treatment have also been observed in rodents following periods of morphologically normal spermatogenesis (194,195). In addition, long-term effects on sperm fertilizing ability have been observed following gestational and lactational exposure to Aroclor 1242 (85) and genistein (Fielden et al. unpublished data). Most studies examining the effects of developmental exposure to endocrine disrupting chemicals on male reproductive health have focused on younger adults. With a few exceptions, the persistence and manifestation of effects at later stages of life have been relatively ignored. Based on the results of this study and others (84,85,165), the long-term effects of synthetic and natural estrogenic chemicals warrant further investigation.

The size of the testis, the number of Sertoli cells, and germ cell production in adulthood are highly correlated with the number of Sertoli cells produced during the perinatal period, which is positively controlled by FSH (86-88). As expected, the relative decrease in Sertoli cell count in the present study (17 and 32 % on PND105 and 315, respectively) was reflected by a comparable decrease in epididymal sperm count (19 and 26%). This supports the hypothesis of Sharpe and Skakkebaek (15) that estrogens can inhibit sperm production in adulthood by decreasing Sertoli cell proliferation and testis size during the perinatal growth phase. The hypothesis is further supported by studies in rats and ewes where maternal estrogen treatment (octylphenol or DES) decreased fetal FSH in the pituitary (196,197). Moreover, gestational and lactational exposure to DES in drinking water has been shown to decrease adult testes weight and sperm production in rats (81,82). This effect, however, may be

specific to DES since *in utero* and postnatal dietary exposure to 17β-estradiol (E2) did not effect the number of Sertoli cells in adult rats (198). Although FSH levels were not measured in this study, the decrease in the number of Sertoli cells in the 10 μg/kg group is consistent with a decrease in perinatal levels of FSH.

The suppression of gonadotropin secretion alone is not likely sufficient to explain the effects on sperm quality. Even after normalizing the number of sperm per dish in the IVF assay, decreases in sperm fertilizing ability were still observed (Table 4). Furthermore, the effects of neonatal DES treatment on Sertoli cell maturation and efficiency of spermatogenesis in adulthood are distinct from those induced by neonatal treatment to a GnRH antagonist, and may involve changes in Sertoli cell gene expression (89,91). Decreases in FSH during the perinatal stage may also have adverse consequences on the multiplication and differentiation of spermatogonia into spermatocytes, and differentiation and maturation of Leydig cells (199-203). Although androgen levels were not measured in this study, the lack of effects on AGD and seminal vesicle weight argue that systemic androgen action, and presumably testicular steroidogenesis, was unaffected. However, gestational exposure to DES decreased testicular and serum testosterone levels in fetal rats in the absence of changes in pituitary LH content (204). Testicular androgen levels are also over a magnitude higher than plasma levels (205), and subtle changes in testicular steroid synthesis may impact spermatogenesis in the absence of changes in plasma steroid levels or changes in AGD and seminal vesicle weight. Androgen signaling may also be

disrupted at the level of the androgen receptor (AR). For example, DES has been shown to antagonize E2-regulated gene expression through the AR (206,207). In addition, DES treatment has been shown to decrease AR expression in the testes and other tissues of the Wolffian duct, albeit at high doses (>10 μg/rat) (208).

The effects on sperm fertilizing ability are difficult to explain in the absence of molecular or biochemical data since the critical effects induced by DES may reside in one of many possible cell types within and outside of the testis; all of which contribute to sperm fertilizing ability. Furthermore, the effects may not be evident histologically or may have occurred during periods of development not examined. The long-term effects on sperm fertilizing ability may indicate that DES is acting as a mutagen and irreversibly changing the genetic program of the germ cells. Under certain conditions, DES has been shown to increase unscheduled DNA synthesis, sister-chromatid exchanges, chromosomal aberrations, aneuploidy and mammalian cell transformation (reviewed in (209)). Although adult exposure to high doses of DES (>50 mg/kg) increases the incidence of micronuclei and sperm-head abnormalities in the adult rat testis (210), prenatal or neonatal administration of DES does not affect micronucleus or sperm-head abnormality rates at doses much higher than in the present study (210). Alternatively, there is evidence supporting long-term epigenetic alterations following developmental exposure to DES, which may involve hypomethylation of estrogen-responsive genes (211,212). Administration of 5-azacytidine, a methylation blocker, to adult rats also caused a decrease in fertility and an

increase in the number of abnormal embryos on GD 2 (213). Abnormal imprinting of DNA methylation patterns has also been proposed as a possible mechanism for the transgenerational effects of DES (214,215). Abnormal imprinting of germ cell DNA during gametogenesis may explain the decrease in sperm fertilizing ability and higher proportion of eggs that did not proceed to the two-cell stage in the 10 μg/kg group on PND105 (Table 4). DES-induced nuclear aberrations and loss of synaptonemal complexes have also been reported in meiotic prophase nuclei of *C. elegens* (216).

Changes in sperm fertilizing ability may also involve changes in estrogen signaling independent of the methylation status of estrogen-responsive genes. The involvement of estrogen signaling is supported by studies of ERα and aromatase knockout mice, which have demonstrated a role for ERα and E2 in controlling sperm fertilizing ability *in vitro* and fertility *in vivo* (37,44). The testicular development and sperm quality of ERβ knockout mice is normal (217). It is interesting to note that the testes of ERα and aromatase knockout mice are histologically normal in prepubertal mice, but exhibit an age-related structural deterioration that progresses to infertility (37,43,44). Prenatal DES exposure also decreases the responsiveness of the uterus to prepubertal estrogen stimulation (218), thus raising speculation that prenatal DES exposure can also reduce the responsiveness of the male reproductive tract to estrogens, thereby causing phenotypic changes comparable to those observed in ERα or aromatase knockout mice. Although estrogens are inhibitory to testicular development at pharmacological doses, they are required for normal fertility at physiological

levels. Therefore, estrogens may have the potential for stimulating spermatogenesis at low doses, as seen with the increase in sperm fertilizing ability in the 0.1 μg/kg group (Table 4). Stimulatory effects of genistein, a weak estrogen agonist, on sperm fertilizing ability have also been observed in offspring exposed through gestation and lactation (Fielden et al. unpublished results). Tamoxifen, a weak agonist in the male reproductive tract (219,220), was also found to increase fertility in adult males treated with low doses, while inhibiting fertility at higher doses (221). Although controversial, studies have reported that low doses of estrogen have a stimulatory effect on prostate weight (79,222). These findings suggest that estrogenic endocrine disruptors can act through multiple mechanisms of -action that are dependent on dose and estrogenic efficacy. Considering the role of the ER as a transcription factor, it is reasonable to expect that the effects of DES involve changes in gene expression, which may include ER (α or β) itself, their downstream target genes, and possibly other unknown target genes. Based on this hypothesis, cDNA microarrays enriched for genes expressed in the testis were constructed in order to examine testicular gene expression in the DES-exposed mice. The results of these studies are reported in Chapter 6.

Non-ER mediated effects must also be considered for DES-induced reproductive toxicity. For example DES, but not E2, can inhibit transcriptional activity of the AR and the estrogen-related receptor (ERR) family of orphan nuclear receptors (187,206,223). It is interesting to note that ERRα homodimers can bind to and activate transcription through an SF-1 response element (224)

while DES has also been shown to inhibit expression of the SF-1 target gene 17α-hydroxylase/C17-20 lyase in fetal rat testes (92,225). In addition, DES is more bioavailable than E2 as a result of lower affinity for serum binding proteins (226). The multiple activities of DES, in addition to the increased biological availability of DES in fetal rodents when compared to E2, may explain the higher potency of DES as an endocrine disruptor in rodents compared to E2 (227,228). Although the epididymis was not examined in this study, DES may also induce long-term effects on epididymal structure and function. For example, *in utero* and continuous postnatal dietary exposure to E2 caused a long-term (to PND 98) decrease in epididymal weight and sperm count, which did not return to control levels after 103 days of recovery (198). Due to the importance of the epididymis in the maturation of spermatozoa and the acquisition of fertilizing ability, investigating the functional consequences of gestational and lactational DES exposure on the epididymis is warranted.

Many studies have reported an increased incidence of urogenital tract abnormalities in human males exposed to DES *in utero* (61,65-69). Impaired semen quality has also been reported in DES exposed males (65,67,68,229), however, others have found little or no association (66,71). A recent study examining the fertility in a large number of men exposed to high doses of DES *in utero* found no evidence of impaired fertility, despite an increase in the incidence of urogenital tract abnormalities in these men (61). It would appear then that genital malformations and decreases in semen quality in humans do not adequately predict changes in fertility, as also observed in rodent studies.

However, studies on human fertility are difficult to interpret since fertility measures are confounded by environmental, behavioral and maternal factors, and therefore do not reflect sperm fertilizing ability. In addition, the clinical data does not address any health effects that might emerge at older ages since most DES-exposed men have not yet reached 50 years old. Considering the long-term effects observed in this study, and the effects of prenatal exposure on prostate development in mice (207,230), it would be of importance to monitor fertility and prostate development in aging men exposed to DES.

In summary, the data described in this report demonstrate the potential for developmental exposure to DES, and possibly other estrogenic chemicals, to irreversibly alter testicular development and sperm function. This is supported by previous studies demonstrating long-term or latent effects on spermatogenesis following developmental exposure to estrogens. Long-term effects following perinatal estrogen exposure have also been observed on bone homeostasis (231-233), mammary gland development (234-237), and reproductive tract development in males and females (238) and those mentioned above). These results, and those of concurrent studies (Chapter 6) highlight the importance and utility of assessing developmental toxicants at multiple levels of biological organization and throughout the life cycle, while also including functional and molecular endpoints to comprehensively assess the molecular changes resulting in physiological alterations.

CHAPTER 6

GESTATIONAL AND LACTATIONAL EXPOSURE OF MALE MICE TO DIETHYLSTILBESTROL: TRANSIENT AND LATENT EFFECTS ON TESTICULAR GENE EXPRESSION ASSESSED BY cDNA MICROARRAYS AND REAL-TIME PCR[4]

ABSTRACT

To investigate the molecular events underlying the adverse effects on sperm quality following developmental exposure to estrogenic chemicals, a testis-enriched mouse cDNA microarray was constructed to examine testicular global gene expression in B6D2F1 male mice exposed to 10 µg/kg diethylstilbestrol (DES) from gestational day 12 to postnatal day (PND) 21. Gene expression was examined in testes collected on PND21, 105 and 315 to determine if the long-term effects on testicular development, epididymal sperm count, and *in vitro* fertilizing ability are associated with changes in testicular gene expression. A cDNA microarray containing 1948 unique expressed sequences was constructed and used to compare replicate gene expression profiles in DES-exposed offspring (labeled with Cy5) to that of age-matched vehicle-exposed animals (labeled with Cy3). An independent reference design coupled with

[4] Submitted for publication in Fielden M.R., Halgren R.G., Fong C.J., Chou K., Zacharewski T.R. (2002) Gestational and lactational exposure of male mice to diethylstilbestrol: II. transient and latent effects on testicular gene expression assessed by cDNA microarrays and real-time PCR.

paired t-tests identified genes putatively altered in expression by DES. Real-time PCR was used to verify differential expression and to examine the dose-dependency of the response. Real-time PCR confirmed that changes in the expression of orphan testicular receptor Tr2-11, inhibin βC, prosaposin/SGP-1, and the homoebox A10 gene were observed concurrently with the long-term changes in testicular development and sperm function. DES also caused transient changes in the expression of the HDL receptor SR-B1 and the lysosomal glycoprotein LGP85/LIMPII on PND21. Real-time PCR demonstrated that steroidogenic factor 1 (SF-1) was significantly decreased by DES on PND21. Consistent with this was a significant decrease in the expression of the SF-1 responsive genes 17α-hydroxylase/C17,20-lyase (Cyp17), cytochrome P450 side chain cleavage enzyme (Cyp11a), and the steroidogenic acute regulatory protein (Star). Although SF-1 expression was not affected on PND105, the expression of Cyp17, Cyp11a and Star were all significantly increased at this time point. Changes in testicular gene expression were most apparent in the 10 μg/kg group, as were the decreases in testicular growth, number of Sertoli cells, epididymal sperm count, and sperm fertilizing ability *in vitro*. By contrast, estrogen receptor (ER) α expression was decreased in a dose-dependent manner on PND21, while the expression of ERβ and the androgen receptor were not altered at any age. The results presented here and in Chapter 5 demonstrate that early exposure to DES causes long-term adverse effects on testicular development and sperm quality, and these effects are associated with transient and latent effects on testicular gene expression, even long after the cessation of

DES exposure. These results also suggest multiple mechanisms by which early developmental exposure to estrogen disrupts estrogen signaling, steroidogenesis, lysosomal function, and testicular development.

INTRODUCTION

Diethylstilbestrol (DES) is a non-steroidal synthetic estrogen that was administered to pregnant women to prevent miscarriages in the late 1940's and 1950's. Its use was terminated in 1971 when it was concluded that prenatal exposure to DES causes vaginal and cervical clear-cell adenocarcinoma in female offspring following otherwise normal pubertal development (55,56). Putative effects in human males include testicular cancer, hypospadias, cryptorchidism, lowered sperm count, and impaired fertility (170). A causal link between estrogen exposure *in utero* and adverse effects on the male reproductive tract and sperm quality is also supported by studies of wildlife populations exposed to estrogenic chemicals in the environment (167). This has raised a concern over exposure to both synthetic and natural estrogenic endocrine disruptors (EEDs) in the environment and their effects on human reproductive health (239). Epidemiological studies supporting a decrease in human sperm count (1,2) and an increase in testicular cancer and reproductive tract malformations (12,14) have also heightened the concern over EEDs. This has subsequently led to an increase in the identification and characterization of other hormone-like chemicals in the environment (51,52). Consequently, DES has been extensively used to characterize the effects of EEDs on reproductive health and to investigate the role of estrogen in male reproductive tract development in various animal models (73,171). For example, *in utero* exposure of male mice to DES causes sterility resulting from a number of reproductive tract

abnormalities, including enlarged and cystic Müllerian remnants, inhibition of gubernaculum development and cryptorchidism, sperm granulomas, hypotrophic testes and epididymides, epididymal cysts of embryonic female origin, and tumors of the rete testis and interstitial cells (74-80,172-174). Non-teratogenic effects induced by DES include adverse effects on testicular development, and decreases in sperm production and sperm fertilizing ability (81,82,240).

The molecular mechanisms responsible for the adverse effects of DES are unclear; however, there is evidence that the initiating events involve changes in gene expression. For example, estrogen-induced cryptorchidism is believed to occur as a result of down-regulation of insulin-like factor 3 in the Leydig cells of the fetal mouse testis (80,174). Incomplete regression of the Müllerian ducts in DES-exposed male fetuses may involve increased expression of steroidogenic factor 1 (SF-1) and anti-Müllerian hormone and its receptor (78). By contrast, expression of SF-1 and its target gene 17 α–hydroxylase/C17,20-lyase (Cyp17) were reported to be decreased in the Leydig cells of DES-exposed fetal rats (92,225). *In utero* DES exposure has also been reported to affect the expression of estrogen receptor (ER)α, Wnt7a, epidermal growth factor, and various Hox genes in the Müllerian duct of female mice (241-246). Estrogens may also exert long-term reprogramming effects by altering gene expression, cellular differentiation and tissue restructuring, and concomitant responsiveness of various tissues of the male reproductive tract (247), thus possibly leading to reproductive tract abnormalities, malfunction of the Wolffian ducts, and adverse effects on spermatogenesis.

The changes in gene expression induced by prenatal exposure to DES are likely to be mediated, at least in part, by the estrogen receptor (ER). The distinct expression pattern and ontogeny of ERα and ERβ in the rodent testis (30,33,34), and the detrimental effect of genetic disruption of ERα signaling (37) and estrogen synthesis (44) highlight the importance of estrogen and its receptor for normal spermatogenesis and sperm fertilizing ability. In addition to its ER-agonist activity, DES also inhibits the transcriptional activity of the estrogen – related receptor (ERR) orphan nuclear receptors (187,223) and binds to the androgen receptor (AR) to inhibit 17β-estradiol (E2)-induced gene expression (206), thus raising speculation that DES may disrupt male reproductive development through mechanisms independent of the ER. In addition, the mutagenicity of DES (209) and the carcinogenicity in reproductive tissues following developmental exposure to DES (76,173,214) suggest that genotoxic mechanisms may contribute to the effects on fertility. Clearly, a more thorough understanding of the molecular changes induced by DES is needed in order to understand the pathophysiology of DES on the male reproductive system.

With the advent of cDNA microarrays, it is now possible to explore the molecular changes that underlie pathological processes in a species and tissue-specific manner. The objectives of this study were to explore the molecular changes in the testes of mice that were developmentally exposed to DES. It is hypothesized that the long-term effects of DES on sperm quality are a result of persistent changes in testicular gene expression. Therefore, cDNA microarrays were used to identify gene expression changes in the testis following gestational

and lactational exposure to DES. Commercially available cDNA microarrays often contain a wide variety of genes involved in diverse cellular activities and/or expressed in many different cell types. As a result, many genes on commercial arrays do not yield sufficient information relevant to the problem or model system of interest. This limitation prompted us to design and construct cDNA microarrays specific for studying gene expression in the murine testis in order to maximize the amount of useful expression data. In this study, pregnant mice were gavaged with vehicle or 0.1, 1, and 10μg/kg/day of DES from gestational day (GD) 12 to postnatal day (PND) 21. Testicular weight, testicular histology, Sertoli cell morphometry, epididymal sperm count, sperm motility and *in vitro* fertilizing ability were assessed in male offspring and reported in Chapter 5. DES was found to cause a long-term decrease in the number of Sertoli cells, epididymal sperm count and *in vitro* fertilizing ability in the absence of gross reproductive tract abnormalities. This chapter reports the results of testicular gene expression analysis using a customized cDNA microarray enriched for genes expressed in the mouse testis, in addition to real-time PCR.

MATERIALS AND METHODS

Animals

All mice were obtained from Charles River Laboratories (Portage, MI) and housed in polycarbonate cages with cellulose fiber chips (Aspen Chip Laboratory Bedding, Northeastern Products, Warrensberg, NY) as bedding and maintained in a humidity (30-40 %) and temperature (23 °C) controlled room on a 12 h light-dark cycle. All animals were given free access to deionized water in glass bottles with rubber stoppers, and AIN-76A rodent feed (Research Diets, New Brunswick, NJ). This diet is a casein-based open-formula purified diet without detectable levels of the estrogenic isoflavones genistein, diadzein or glycitein (unpublished data and (175,176)).

F0 treatment

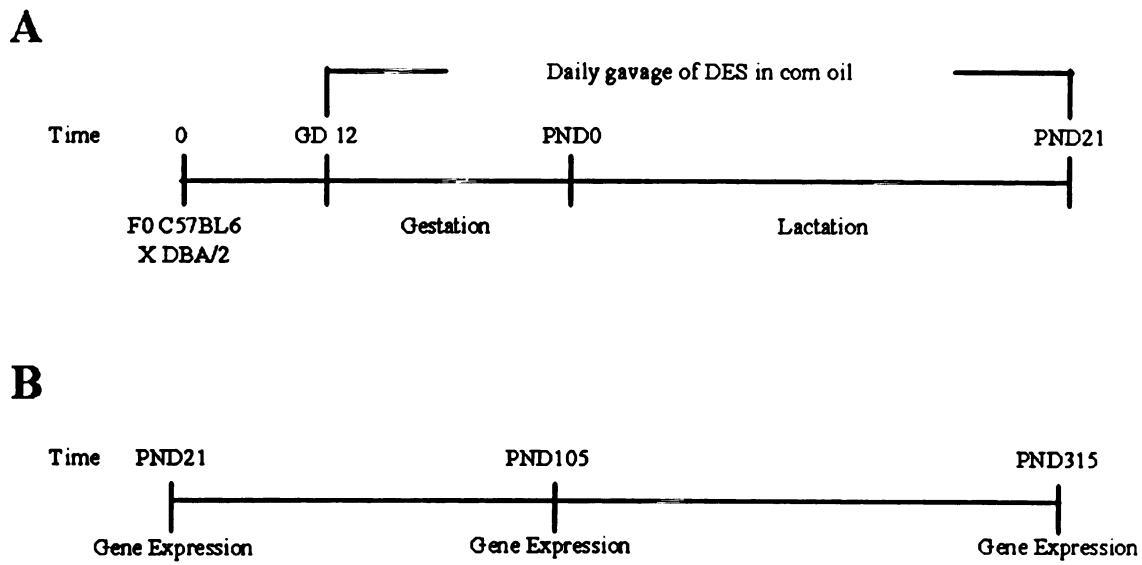
Details of the F0 treatment are described in Chapter 5. Briefly, mature virgin 11-week old C57BL/6 female mice (F0) were housed two per cage upon arrival and acclimatized for at least three days. Each pair of females was housed with a 7- to 8-week old DBA/2 proven breeder male within a week of arrival. Offspring from this cross are designated B6D2F1. Following evidence of pregnancy, dams were separated from the males, housed individually, and consecutively assigned to one of four treatment groups. F0 mice were treated by daily gavage with 0.1 ml of corn oil (vehicle; Sigma, St. Louis, MO) or a nominal dose of 0.1, 1, or 10 µg DES per kg of maternal body weight (09:00 – 12:00). Treatment was performed from gestational (GD) 12 to postnatal day (PND) 20,

with a one-day interruption of treatment on the day of parturition (PND0). The dose of test chemical was adjusted daily to body weight for each mouse prior to dosing. Offspring were weaned on PND21 and housed with same sex littermates. F0 mice were euthanized on PND21. The F0 treatment and F1 necropsy schedules are shown in Figure 1.

F1 necropsy and testicular gene expression

F1 male offspring were necropsied on PND21, 105, or 315 and body weight, anogenital distance, testicular growth, testicular histology, Sertoli cell morphometry, epididymal sperm count and motility, and *in vitro* fertilizing ability were assessed. The results are reported in Chapter 5. Here we report the results of testicular gene expression studies using cDNA microarrays and real-time PCR. To identify genes differentially expressed between treatment groups, an independent reference design was used. This design is based on quantitating gene expression in replicate independent samples from a treated group (i.e. 10 µg/kg DES) and comparing them to a reference group (i.e. vehicle control). cDNA microarrays were used to profile gene expression differences in the testis of male B6D2F1 offspring in the 10 µg/kg DES group relative to offspring in the vehicle control group on PND21, 105 and 315 (Figure 1). The 10 µg/kg DES group was chosen for comparison against vehicle control animals since changes in testicular development and sperm quality were demonstrated at this dose (Chapter 5). Each microarray experiment (i.e. hybridization) compared the relative level of gene expression for a single animal in the DES group (Cy5-labeled cDNA) to that of a single animal in the control group (Cy3-labeled cDNA)

Figure 1. Treatment schedule for F0 mice and necropsy schedule for F1 male offspring. A) F0 C57BL/6 mice were fed a soy-free diet and mated with a male DBA/2 mouse. Pregnant mice were dosed daily by gavage with 0.1 ml of corn oil (vehicle) or 0.1, 1, and 10 µg DES per kg of maternal body weight from gestational day (GD) 12 to postnatal day (PND) 21. B) Male B6D2F1 offspring were weaned on PND 21 and housed with same-sex littermates. F1 males were necropsied for testicular gene expression on either PND 21, PND 105 (15 weeks of age), or PND 315 (45 weeks of age). In addition, male offspring were assessed for body weight, anogenital distance, testicular growth, testicular histology, Sertoli cell morphometry, and epididymal sperm count, motility, and *in vitro* fertilizing ability.


A
Daily gavage of DES in corn oil
Time
0
GD 12
PND0
PND21
F0 C57BL6
X DBA/2
Gestation
Lactation
B
Time
PND21
PND105
PND315
Gene Expression
Gene Expression
Gene Expression

within each age. One animal per litter was used for the control and DES groups such that replicate gene expression measurements could be made on independent experimental units (i.e. the litter) on PND21 (n=5), PND105 (n=9), and PND315 (n=4). Differentially expressed genes were then identified at each of the three time points by paired t-tests and p values that were adjusted by the step-down Bonferroni method (described below). Both raw and adjusted p values were used to prioritize genes that were putatively considered to be differentially expressed between control and DES-exposed animals. Genes on the microarray were selected for experimental verification by real-time PCR based on p values, the persistence of the putative change in gene expression over time, and their possible role in spermatogenesis based on functional information from the literature. Additional genes not on the array were also quantitated by real-time PCR as described below.

Testicular RNA isolation

One testis per animal was immersed in five volumes of RNALater (Ambion, Austin, TX) in a 2 ml tube and immediately placed on dry ice before long-term storage at –20°C. For total RNA isolation, tissue frozen in RNALater was thawed on ice and transferred to 0.75 ml of Trizol Reagent (Life Technologies, Rockville, MD). Tissue was then immediately homogenized and total RNA isolated as described in the manufacturer's instructions. Linear acrylamide (20 µg) was added as a carrier to aid precipitation and increase yield. RNA pellets were resuspended in 25 to 50 µl of 1 mM sodium citrate pH 6.4 and

stored at –20°C until use. Total RNA was quantitated (A_{260}) and assessed for purity (A_{260}/A_{280}) by spectrophotometry.

Microarray design strategy

To maximize the amount of useful gene expression information and increase the probability of detecting relevant changes in testicular gene expression, a list of genes expressed in the testis was generated and used to select genes to be printed on the microarray. Mouse sequences were included in the list if they: 1) had been experimentally determined to be expressed in the mouse testis, 2) had been cloned from a mouse testis cDNA library, or 3) were putative orthologs of rat or human genes known to be expressed in the testis. Experimental data demonstrating that a gene was expressed in the mouse testis was collected from the literature (PubMed; www.ncbi.nlm.nih.gov/entrez) using key word searches, or from gene expression data in the Jackson Laboratory Gene Expression Database (www.informatics.jax.org/mgihome/GXD). Preliminary cDNA microarray data using a variety of commercial arrays were also used to experimentally determine what genes are expressed in the adult testis (data not shown). Sequence information on known genes or expressed sequence tags (ESTs; considered to have a putative identity based on sequence similarity to genes of known identity in other species) that were derived from cDNA libraries of mouse testicular origin were obtained from the NCBI Unigene database (www.ncbi.nlm.nih.gov/UniGene). Putative mouse orthologs of rat and human genes known to be expressed in the testis (determined from PubMed searches) were also included. Putative orthologs were chosen by selecting the

best sequence match with the lowest E-value (with an arbitrary cutoff of $< 10^{-39}$) from a TBLASTX search of mouse dbEST (www.ncbi.nlm.nih.gov/dbEST). Genes included on the microarray were chosen from the list if a corresponding cDNA clone representing the gene was commercially available from the IMAGE consortium collection (248). Priority was given to those genes that had functional information. A total of 1189 cDNA clones were selected and purchased from Research Genetics (Huntsville, AL) and sequence verified at the Michigan State University Genomic Technology Support Facility (www.genomics.msu.edu). This resulted in 1304 unique sequence verified cDNA clones. The additional 115 unique cDNA clones were physically isolated from stock cultures contaminated with more than one unique plasmid, as described (249). Another 984 sequence verified cDNA clones were also obtained from Research Genetics for a total of 2288 unique cDNA clones (Kindly provided by Dr. David Dix, U.S. Environmental Protection Agency, NC, through the E.P.A. Microarray Consortium (250). The accession number of each cDNA clone was queried against the mouse UniGene database to assign a putative gene identity. Based on build 99 of UniGene, 1873 cDNA clones have been assigned a gene name, 334 are ESTs, and 97 have no match to any gene in the mouse UniGene Database. In total, 2304 cDNA clones (2288 unique) representing 1948 unique UniGene clusters were used in the construction of the microarray.

Microarray construction

cDNA inserts were amplified from ~100 ng of plasmid DNA in a 100 µl PCR reaction in Microamp 96-well Reaction Plates (Applied Biosystems, Foster

City, CA). The reaction mixture contained 1x PCR buffer (20 mM Tris-HCl, 50 mM KCl, pH 8.4), 0.2 mM dNTPs, 3 mM $MgCl_2$, 5 units of Taq polymerase (Life Technologies, Rockville, MD), and 0.4 µM each of forward T7 primer (5'-TAACCCTCACTAAAGGGA-3') and reverse T3 primer (5'-TAATACGACTCACTATAGGG-3'). The primers were modified with primary amines on the 5' end to facilitate covalent coupling to the aldehyde-derivatized glass slides. The PCR cycling conditions were as follows: initial denaturation for 1 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 56°C, 2 min at 72°C, and a final elongation of 7 min at 72°C. To confirm amplification, all PCR products were electrophoresed on a 1% agarose gel containing 0.4 µg/ml ethidium bromide. Failed amplifications were repeated until 100 % of the clones produced a PCR product detectable by electrophoresis.

PCR products were precipitated directly in the PCR plate by the addition of 200 µl ice cold 100% ethanol and 3 M ammonium acetate (95:5) for 1 h. DNA was pelleted by centrifugation at 4°C at 2200 x g for 45 min. The supernatant was discarded and the DNA pellet was washed with 200 µl of ice cold 75% ethanol. Following centrifugation at 2200 x g for 30 min at 4°C, the supernatant was discarded and the DNA pellet was air dried. The DNA was resuspended in 10 µl of 1x spotting solution (Telechem, Sunnyvale, CA), transferred into 384-well plates (DOT Scientific, Burton, MI), sealed with adhesive foil, and stored in a sealed bag at –20°C until printing.

DNA was robotically arrayed onto Superaldehyde slides (Telechem) using an Omnigrid arrayer (GeneMachines, San Carlos, CA) equipped with 16 (4x4)

Chipmaker 2 pins (Telechem). Each spot on the array was printed in duplicate. Humidity was maintained at 55 % during printing. Printed slides were denatured and blocked according to the manufacturer's instructions (Telechem) and stored at room temperature.

cDNA synthesis and hybridization

Total RNA (25 µg) was used as template for a reverse transcriptase reaction in 30 µl of 1x First Strand Synthesis buffer (Life Technologies) containing 6 µg oligo(dT_{18}A/C/GN), 417 µM dATP, dCTP, and dGTP, 167 µM dTTP, 2 nmol Cy5- (DES-exposed animals) or Cy3-dUTP (vehicle control animals) (Amersham Pharmacia, Piscataway, NJ), 10 mM DTT, and 400 units of SuperScript II reverse transcriptase (Life Technologies). The reaction mixture was incubated at 42°C for 60 min. The reaction was stopped by the addition of 10 µl of 0.1 M EDTA. RNA was hydrolyzed by the addition of 15 µl of 0.1 M NaOH and incubation at 70°C for 10 min. The reaction was neutralized by the addition of 15 µl of 0.1 M HCl.

The fluor-labeled cDNA was purified with the Qiagen PCR Purification Kit (Qiagen, Valencia, CA) and eluted in 50 µl of water. The Cy3-labeled cDNA was mixed with the Cy5-labeled cDNA, vacuum dried, and resuspended in 32 µl of hybridization buffer (40% formamide, 4x SSC, 1% SDS) containing 20 µg of polyA and 20 µg of mouse COT-1 DNA (Life Technologies) as competitor. The probe mixture was heated at 95°C for 2 min before being hybridized to the microarray under a 22x40 mm cover slip (Corning Life Sciences, Corning, NY) in a light protected humidified hybridization chamber (Corning Life Sciences). The

microarray was hybridized for 16 to 18 h at 42°C. Slides were washed at room temperature in 1x SSC, 0.1% SDS for 5 min, 0.1x SSC, 0.1% SDS for 5 min, and twice in 0.1x SSC for 1 min. The slides were dried by centrifugation at 500 rpm for 5 min and scanned promptly. Protocols can also be found at http://www.bch.msu.edu/~zacharet/microarray/microarray.html.

Image acquisition and data processing

Slides were scanned at 635 nm (Cy5) and 532 nm (Cy3) on an Affymetrix 428 Array Scanner (Santa Clara, CA). Images were analyzed using GenePix 3.0 (Axon, Foster City, CA). Blocks were manually adjusted and features automatically detected and quantitated as suggested in the manufacturer's instructions. Images were surveyed visually to flag anomalous spots. GenePix results files (i.e. gpr files), which contain raw signal intensity and background signal intensity values for each spot and channel, were further processed in batch using an automated and customizable script called GP3. GP3 filters spots below the limits of detection or at saturating levels, corrects for background signal, and applies a global linear normalization factor in log space in order to generate valid estimates of gene expression in each channel (Chapter 7). Details of the algorithm, and downloads can be found at http://www.bch.msu.edu/~zacharet/microarray/GP3.html.

Statistical analysis of microarray data

To assess the degree of experimental, biological and treatment-induced variation in gene expression measurements, Pearson correlation coefficients (r) were calculated to compare the similarity between sample measurements within

and across hybridizations (GraphPad Prism 3.0, San Diego, CA). Correlation coefficients were normalized with a Fisher's z' transformation in order to calculate 95 % confidence intervals surrounding r. Differentially expressed genes were identified by computing t-statistics and p values using SAS version 8.0 (Cary, NC). Since paired samples (control and treated) are hybridized on the same array, the assumption of independence is violated and differences cannot be analyzed with a two-sample t-test. Therefore, a paired t-test was used. The paired t-test compares the mean of the differences in the observations according to the null hypothesis that the mean difference equals zero. The t-statistic is given by the equation

$$t_i = \frac{d_i - 0}{\sigma_i / \sqrt{n}}$$

where d is the mean difference between the normalized signal intensity for gene i in channels 1 and 2 (i.e Cy5 and Cy3), σ is the standard deviation of the paired differences and n is the number of paired samples. The significance of the t-statistic was assessed by calculating p values. In order to control the family-wise type 1 error rate the p values were adjusted using the stepdown Bonferroni method (251). The stepdown Bonferroni method adjusts p values by multiplying the nominal p value by the number of comparisons (i.e. number of genes) with equal or lesser test statistics.

Real-time PCR

To verify changes in gene expression observed by microarray analyses, real-time PCR was performed on an Applied Biosystems Prism 7700 Sequence

Detection System (Foster City, CA). Additional genes of interest not on the microarray were also analyzed by real-time PCR (Table 1). The expression level for each gene was measured in all dose groups on PND21, 105 and 315 using six animals per dose group, for a total of 72 animals. To synthesize cDNA, total RNA (1 µg per animal) was used as template for a reverse transcriptase reaction in 20 µl of 1x First Strand Synthesis buffer (Life Technologies) containing 1 µg oligo(dT_{18}A/C/GN), 0.2 mM dNTPs, 10 mM DTT, and 200 units of Superscript II reverse transcriptase (Life Technologies). The reaction mixture was incubated at 42 ºC for 60 min. The reaction was stopped by incubation at 75 ºC for 15 min. Amplification of cDNA (1/20) was performed using the SYBR Green PCR Core Reagents (Applied Biosystems) as suggested by the manufacturer's instructions. All 72 samples were amplified in a single MicroAmp Optical 96-well Reaction Plate (Applied Biosystems). Primer pairs for each gene were designed using Primer3 (252). Gene names, accession numbers, the forward and reverse primer sequences, amplicon size, and optimal primer and Mg^{++} concentrations are listed in Table 1. Amplicon size and reaction specificity were confirmed by agarose gel electrophoresis. In addition, a number of stock cultures of the cDNA clones representing the genes on the microarray were used as a template in a PCR reaction to verify gene-specific amplification, clone identity and the integrity of the clone collection. The PCR cycling conditions were as follows: initial denaturation and enzyme activation for 10 min at 95ºC, followed by 40 cycles of 95ºC for 15 s and 60ºC for 1 min. Each plate contained duplicate standards of

Table 1. Genes, primer sequences, amplicon size and optimal amplification conditions for real-time PCR.

Gene	Accession	Forward Primer	Reverse Primer	Product Length (bp)	Primer Concentration (μM)	Mg2+ Concentration (mM)
Housekeeping genes						
rS14	NM_020600	acgtacgtacgtacgtacgt	acgtacgtacgtacgtacgt	125	0.4	2
β-actin	M12481	gctacagcttcaccaccaca	tctccagggaggaagaggat	123	0.4	2
Genes identified by microarray analysis						
Tr2-11	NM_011629	tgggcactttccataccttc	gttgccacgttcatcacttg	128	0.4	2
Inhbc	NM_010565	ccttccaaagaaaggctcatc	agggaatgtccaaccaatga	126	0.4	2
SR-B1	NM_016741	agcagcaggtgctcaagaat	cttcgtttgggttgaccaat	126	0.4	3
LGP85	NM_007644	tgttgaaacgggagacatca	tggtgacaaccaaagtcgtg	125	0.4	3
Psap	NM_011179	tgaggtgtgcaagaaactgg	tgcttctggtaagggtctgg	124	0.4	2
Hoxa10	NM_008263	gtgtctggcctgaggtcaat	ttggccaaggaagaaaagaa	128	0.4	3
CD36	NM_007643	gcttgcaactgtcagcacat	gccttgctgtagccaagaac	134	0.4	2
Xmr	NM_009529	tgggtgataccaaaggatgg	gtgaggcggcatattctcat	123	0.4	3
Zpr3	NM_009581	atccattccgaaatcaccaa	gattgatcaccactgccaga	146	0.4	2
Bad	NM_007522	cctctcctgttctggactgc	cacatgcgcaggctttatta	122	0.4	2
Sparc	NM_009242	ctgcgtgtgaagaagatcca	ccagtggacagggaagatgt	120	0.4	2
Other genes selected for verification						
ERα	NM_007956	ccgcaagcttattcccttta	ggcgattaagttgggtaacg	105	0.4	2
ERβ	NM_010157	agaatctcttcccagcagca	gggaccacatttttgcactt	125	0.4	3
AR	NM_013476	ggatgggctgaaaaatcaaa	ggagcttggtgagctggtag	134	0.4	2
SF-1	NM_008050	tattacggggtgggaagtca	gtccaaaccccagcacttta	124	0.4	2
Cyp17	NM_007809	atttacccttcggagctggt	agggcagctgtttgtcatct	129	0.8	3
Cyp11a	NM_019779	ttggttccactcctcaaagc	ccaaagtcttggctggaatc	127	0.4	2
Star	L36062	tgctaaggatcgggaactgt	tctggccttttacagaggaga	122	0.4	2

purified PCR products with known template concentration covering at least 6 orders of magnitude in order to interpolate relative template concentration of the experimental samples from standard curves of log copy number versus threshold cycle (C_t). No-template controls (NTC) were also included on each plate. Experimental samples having ā C_t value within two standard deviations of the mean C_t value for the NTC were considered below the limits of detection and assigned a value equal to the mean NTC. The relative level of mRNA was standardized to the housekeeping genes β-actin and ribosomal protein S14 (rS14) in order to control for differences in RNA loading, quality and cDNA synthesis. There were no differences in the results when the data were standardized to either β-actin or rS14 alone. Since the gene expression data was found to violate the assumptions of normality and homogeneity of variance (data not shown), a non-parametric analysis of variance was used to analyze differences in gene expression between control and treated groups. Differences between treated groups and their time-matched control were determined by the Kruskal-Wallis test. Analyses were performed using SAS version 8.0. The level of significance was set at $\alpha = 0.05$. For graphing, the relative expression level was scaled such that the expression level of the time-matched control group was equal to 100.

RESULTS

Experimental variation in gene expression measurements

To assess the reproducibility of the cDNA labeling reaction and the effectiveness of normalization to remove the systematic dye bias, a homotypic hybridization was performed using the same adult testis RNA sample labeled with either Cy3 or Cy5. To quantitate the degree of variation between sample measurements, a Pearson correlation coefficient (r) was calculated. The higher the correlation, the more similar the sample measurements are to each other and the higher the reproducibility of the labeling reaction. A scatter plot of the normalized Cy3 signal versus the normalized Cy5 signal illustrates the reproducibility of the cDNA labeling reaction (Figure 2A). Comparison with the scatter plot of the raw Cy3 signal versus the raw Cy5 signal (Figure 2B) illustrates the effectiveness of the normalization in removing the systematic dye bias. The homotypic hybridization was repeated five times. The average (±SD) correlation coefficient was 0.97 ± 0.01, indicating a high degree of reproducibility in the cDNA labeling reaction.

To assess the reproducibility of replicate spots within each array, a heterotypic hybridization was performed twice, on different days, using control testis RNA labeled with Cy5 and adult kidney RNA labeled with Cy3. The $\log_2$ ratios from duplicate spots within the array were plotted against each other to illustrate the degree of reproducibility between the two ratio measurements

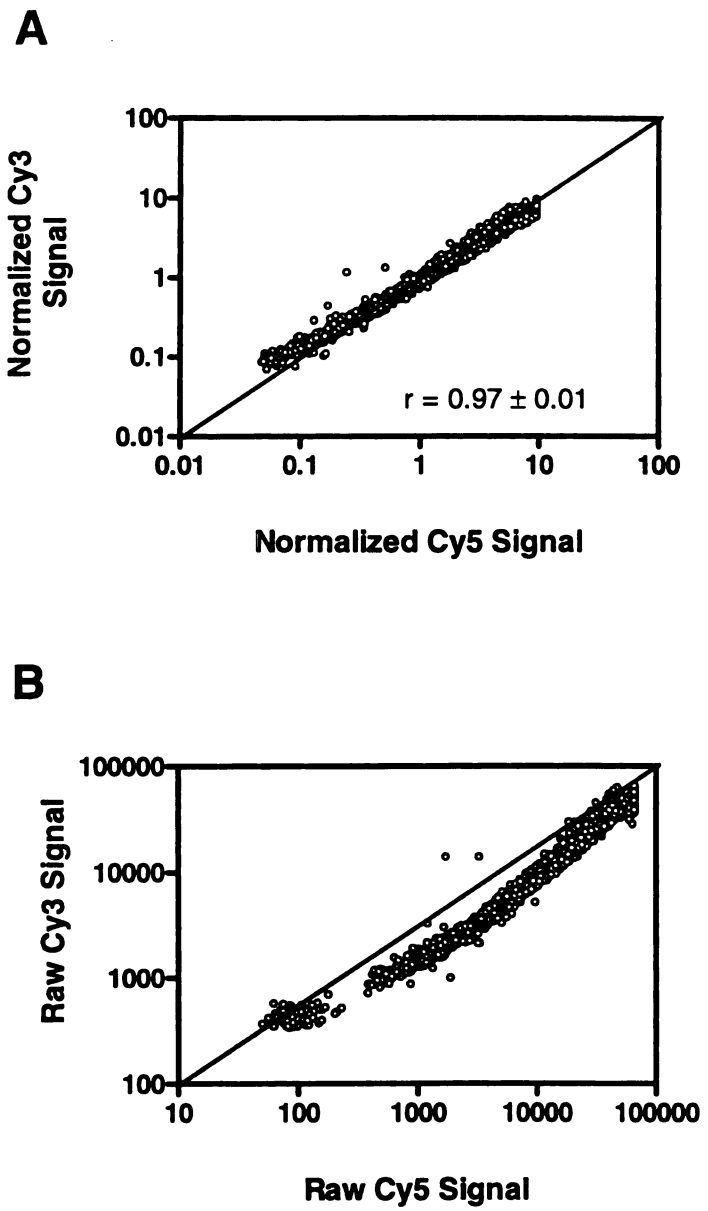


Figure 2. Reproducibility of cDNA synthesis and effectiveness of normalization. A) Scatter plot of normalized Cy3 and Cy5 signal. The same testis RNA sample was labeled with both Cy3 and Cy5 and hybridized to a single array. Raw signal intensity values were background corrected, filtered to remove invalid spots and normalized using a global mean approach, as described in the Materials and Methods. The graph depicts data from a representative experiment that was repeated five times. B) Scatter plot of the raw unnormalized Cy3 and Cy5 signal intensity values from the same experiment plotted in A.

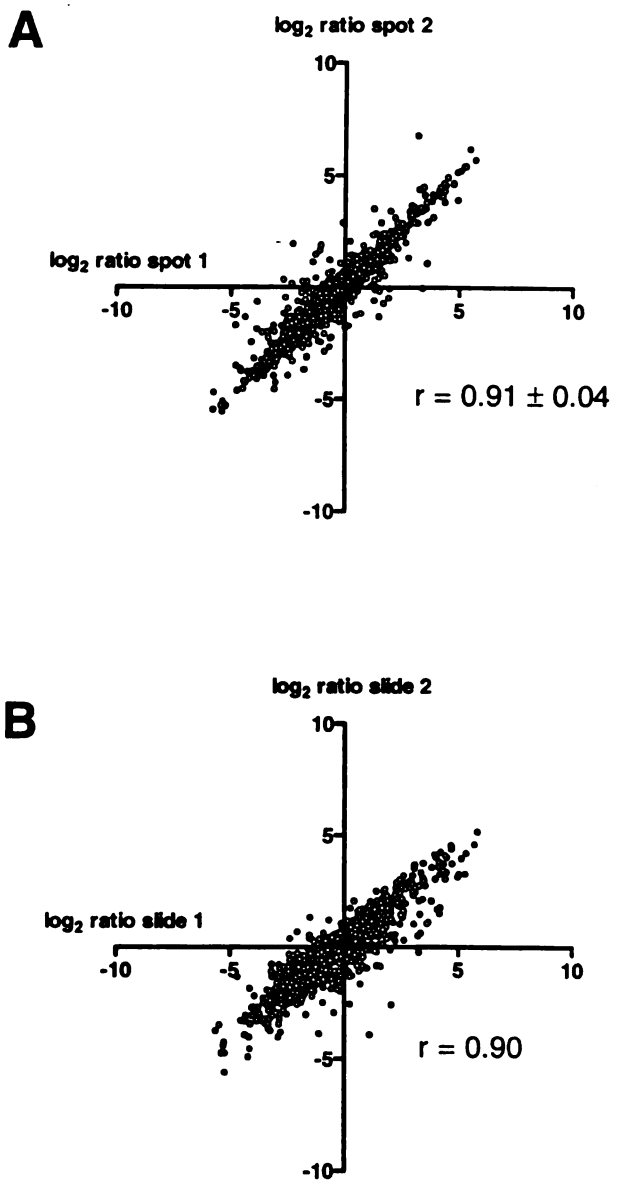


Figure 3. Intraassay and interassay variation in heterotypic hybridizations. A) Scatter plot of $\log_2$ ratio values from duplicate spots within an array. The array was hybridized with cDNA synthesized from kidney (Cy3) and testis (Cy5) RNA. The graph depicts data from a representative experiment that was repeated twice. B) Scatter plot of average $\log_2$ ratio values from two different arrays (from A) that were both hybridized with cDNA synthesized from kidney (Cy3) and testis (Cy5) RNA.

(Figure 3A). The average (±SD) correlation coefficient between replicate spot ratio measurements within the array was 0.91 ± 0.04 (n=2). To assess the reproducibility of gene expression measurements between arrays, the average $\log_2$ ratio values from the two heterotypic hybridizations were plotted against each other to illustrate the degree of reproducibility between ratios measured on different arrays (Figure 3B). The correlation coefficient was 0.90, which indicate that the reproducibility of ratio measurements between arrays is comparable to ratio measurements within arrays.

Biological and treatment-induced variation in gene expression

To assess the degree of biological variation in gene expression, a heterotypic hybridization was performed using testis RNA from two 15 week old control animals, each from different litters. The heterotypic hybridization was repeated five times using 10 different control animals. A representative scatter plot of the normalized Cy3 signal versus the normalized Cy5 signal illustrates the high degree of relatedness between the two samples (Figure 4A). The average (±SD) correlation coefficient was 0.94 ± 0.05 (n=5), which was lower than the average correlation coefficient of the homotypic hybridizations (Figure 2A). This suggests that there was some degree of variation in gene expression between animals that was greater than experimental variation alone. To quantitatively compare the correlation coefficients, a Fisher's z' transformation was used to normalize the correlation coefficients and create 95% confidence intervals for r. The 95 % confidence interval for the homotypic hybridizations ($0.966 \leq r \leq 0.971$; Figure 2A) was greater than the 95 % confidence interval for the control versus

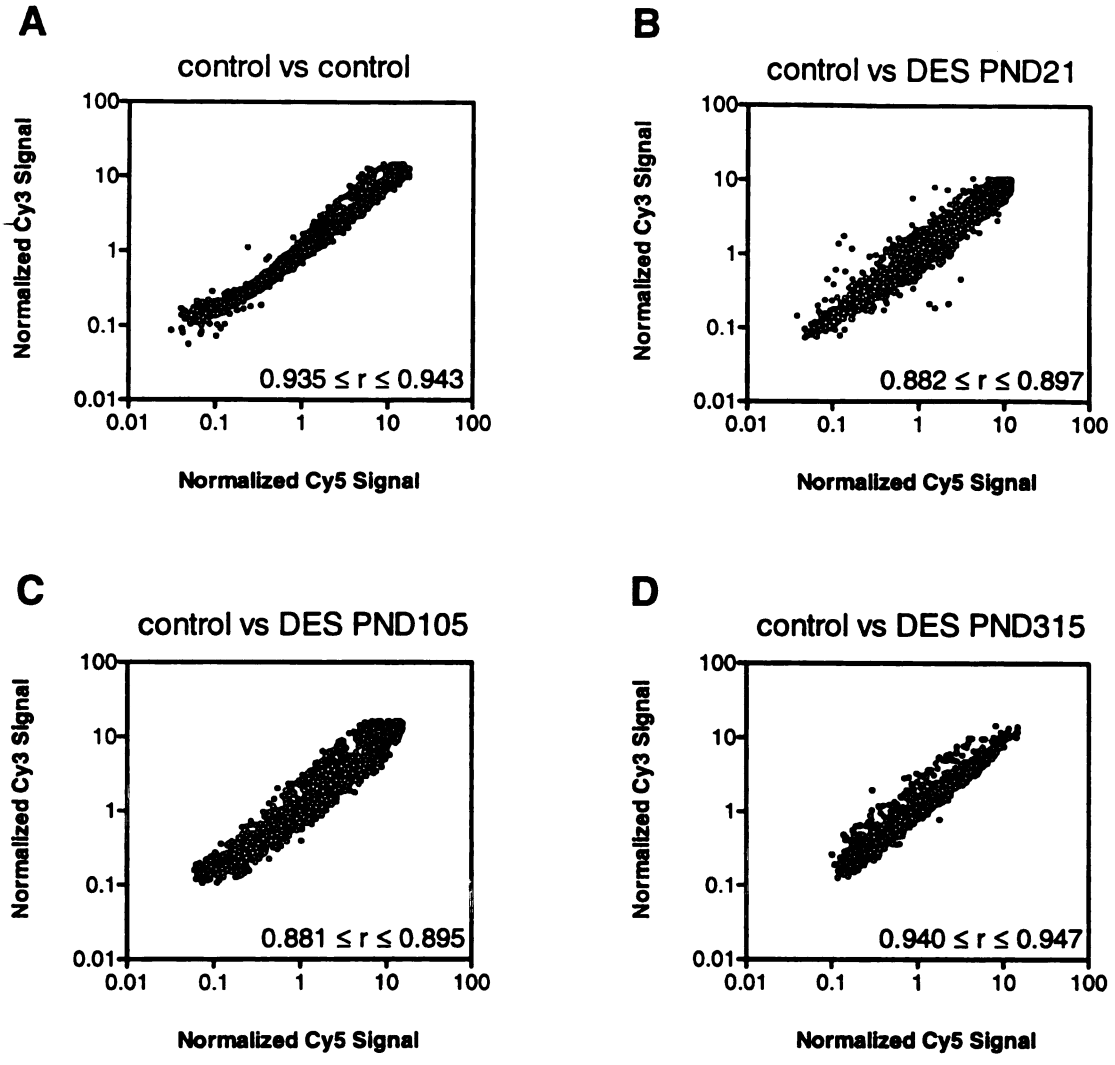


Figure 4. Biological and treatment-induced variation in testicular gene expression. Scatter plots are representative graphs from replicate heterotypic hybridizations comparing gene expression in A) control versus control animals on PND105 (n=5), B) control versus DES animals on PND21 (n=5), C) control versus DES animals on PND105 (n=9), D) control versus DES animals on PND315 (n=4). Correlation coefficients (r) from each hybridization were averaged and used to calculate 95 % confidence intervals for r.

control hybridizations ($0.935 \leq r \leq 0.943$, Figure 4A), thus confirming that animal variation is larger than the experimental variation.

This exploratory approach was extended to determine if DES treatment had an effect on testicular gene expression. Correlation coefficients were calculated for each control versus DES hybridization on PND21, 105 and 315. It was reasoned that an average correlation coefficient lower than that observed in control versus control hybridizations was evidence for a treatment-related effect on testicular gene expression. Comparison of the scatter plots and the 95 % confidence intervals for Pearson correlation coefficients (r) for control (normalized Cy3) versus DES (normalized Cy5) hybridizations (Figure 4B-D) with that of control versus control hybridizations (Figure 4A) illustrates the larger variation in gene expression due to treatment on PND21 and 105, but not PND315. This exploratory analysis suggests that DES significantly affected testicular gene expression on PND21 and 105, but not PND315, at least for the genes represented on the array. To further test this hypothesis and to determine the gene-specific sources of variation, paired t-tests were performed.

Paired t-tests identify genes putatively altered by DES exposure

Test-statistics were calculated for each gene on PND21, 105 and 315 and p-values were used to prioritize genes for verification by real-time PCR. The results of the paired t-tests are summarized in Figure 5. On PND21, there were 644 genes with raw p-values less than 5 %, however, when the p-values were adjusted to control the type 1 error rate, the expression of only one gene was significantly altered by DES ($p<0.05$). This gene was proteasome subunit $\alpha 4$,

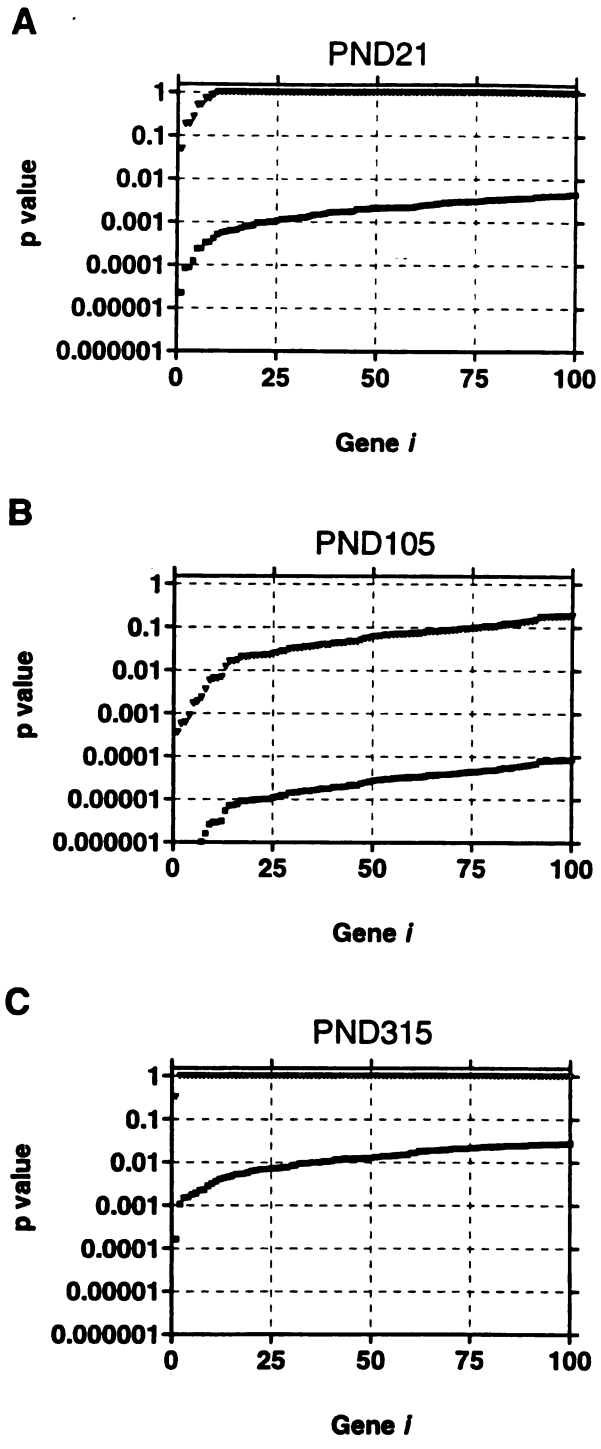


Figure 5. Results of paired t-tests and comparison of raw and adjusted p values. The top 100 ranking genes were plotted against their corresponding raw (■) and adjusted (▽) p values on A) PND21, B) PND105 and C) PND315. P values were adjusted using the stepdown Bonferroni method (251).

which was repressed an average of 1.7-fold (log_2ratio = 0.59 ± 0.16, n=5). On PND105, 1257 genes with raw p-values less than 5 % were identified. Following p-value adjustment, 46 genes were found to be significantly altered by DES ($p<0.05$) (Table 2). Of the 46 genes listed in Table 2, only two were induced by DES (transcobalamin 2 and Duffy blood group) while the other 44 genes were repressed. By contrast, the results of paired t-tests on the control versus control heterotypic hybridizations from PND105 revealed only three genes (EST, calmodulin, IL-2 receptor gamma all induced) that were significantly altered in expression (data not shown). On PND315, there were 178 genes with raw p-values less than 5 %. No genes were significantly altered in expression when the p-values were adjusted for multiplicity. This is consistent with the correlation analysis which suggested that there was no treatment-related effects on gene expression on PND315 (Figure 4), at least for the genes represented on the array. However, other critical but unknown genes may be altered on PND315 since significant changes in Sertoli cell number, epididymal sperm count and sperm fertilizing ability *in vitro* were observed at this age (Chapter 5).

It was hypothesized that the long-term effects of DES on sperm quality were due to transient or persistent changes in testicular gene expression. As an exploratory approach to determine if there were any genes that showed evidence of long-term changes, all genes with raw p values less than 5 % on PND21, 105 and 315 were cross-referenced. This resulted in 32 genes that were putatively altered on both PND21, 105 and 315 (Table 3). Overall, there were 12 of 32 genes that were in agreement with respect to their direction of change at all time

Table 2. Genes significantly altered in expression in the testis on PND105 following gestational and lactational exposure to diethylstilbestrol.

Accession	Symbol	Name	Log_2 Ratio ± SE[a]	Raw p	Adjusted p[b]
AA000473	Evx2	even skipped homeotic gene 2 homolog	-1.46 ± 0.11	1.63E-07	0.0004
AA003599	Ptpn2	protein tyrosine phosphatase, non-receptor type 2	-0.71 ± 0.06	2.60E-07	0.0006
AA220699	Tcn2	transcobalamin 2	0.28 ± 0.03	2.77E-07	0.0006
AA030995	Cbx5	chromobox homolog 5 (*D. Melanogaster* HP1a)	-1.37 ± 0.13	4.00E-07	0.0009
W98547	Bad	Bcl-associated death promoter	-1.18 ± 0.13	7.65E-07	0.0017
AA396114	Net1a	guanine nucleotide regulatory protein (oncogene)	-1.29 ± 0.08	8.55E-07	0.0019
AA167918	-	RIKEN cDNA 1010001N11 gene	-1.24 ± 0.11	1.05E-06	0.0023
AA967055	Fig1	interleukin-four induced gene 1	-1.11 ± 0.12	1.64E-06	0.0036
AA016759	Mcmd6	mini chromosome maintenance deficient 6 (*S. cerevisiae*)	-0.49 ± 0.04	2.64E-06	0.0058
W46030	Sparc	secreted acidic cysteine rich glycoprotein	-0.62 ± 0.08	2.95E-06	0.0065
AA166544	Fech	ferrochelatase	-0.29 ± 0.05	3.00E-06	0.0066
AA437749	Hdh	Huntington disease gene homolog	-0.85 ± 0.04	3.18E-06	0.0070
W65025	jmj	jumonji	-1.12 ± 0.09	5.55E-06	0.0122
AA028786	Mea1	male enhanced antigen 1	-1.33 ± 0.13	7.27E-06	0.0159
AW322547	Fxr1h	fragile X mental retardation gene, autosomal homolog	-0.69 ± 0.05	7.44E-06	0.0163
AA066878	Acta1	actin, alpha 1, skeletal muscle	-0.52 ± 0.05	7.90E-06	0.0173
AI019837	Lamb3	laminin, beta 3	-0.35 ± 0.03	9.34E-06	0.0204
AA003085	Hnrph1	heterogeneous nuclear ribonucleoprotein H1	-0.35 ± 0.04	9.39E-06	0.0205
AW413176	Silg41	silica-induced gene 41	-1.01 ± 0.10	9.70E-06	0.0212
AA458178	Cd36	CD36 antigen	-0.76 ± 0.07	9.89E-06	0.0216
AA290052	Oas1a	2'-5' oligoadenylate synthetase 1A	-0.53 ± 0.05	1.01E-05	0.0221
AA209017	Degs	degenerative spermatocyte homolog (*D. Melanogaster*)	-0.82 ± 0.10	1.02E-05	0.0223
AA254042	Cd3g	CD3 antigen, gamma polypeptide	-0.62 ± 0.07	1.04E-05	0.0227
AI037401	Abi1	abl-interactor 1	-0.59 ± 0.06	1.04E-05	0.0227
AA137833	Supt5h	suppressor of Ty 5 homolog (*S. cerevisiae*)	-0.97 ± 0.06	1.13E-05	0.0247
AA413622	Ei24	etoposide induced 2.4 mRNA	-1.43 ± 0.09	1.17E-05	0.0256

Table 2 (cont'd)

AA510124	Scyb10	small inducible cytokine B subfamily (Cys-X-Cys), member 10	-0.41 ± 0.06	1.27E-05	0.0277
AA403437	Nr2c2	nuclear receptor subfamily 2, group H, member 2	-0.86 ± 0.05	1.28E-05	0.0279
AA137633	Png	phospholipase c neighboring	-0.77 ± 0.08	1.46E-05	0.0317
AA266438	Dfy	Duffy blood group	0.45 ± 0.04	1.49E-05	0.0324
AA000102	Phb	prohibitin	-1.00 ± 0.10	1.50E-05	0.0327
AA166345	Gla	galactosidase, alpha	-1.15 ± 0.13	1.57E-05	0.0342
W61996	Pafah1b1	platelet-activating factor acetylhydrolase, isoform 1b, beta1 subunit	-1.41 ± 0.17	1.63E-05	0.0353
W89549	Epb7.2	erythrocyte protein band 7.2	-1.17 ± 0.12	1.63E-05	0.0354
W88036	Nid1	nidogen 1	-1.18 ± 0.10	1.68E-05	0.0364
AA061388	Xmr	Xlr-related, meiosis regulated	-1.40 ± 0.15	1.73E-05	0.0376
AA636231	Hoxa10	homeo box A10	-1.35 ± 0.15	1.81E-05	0.0393
AA289606	Elk3	ELK3, member of ETS oncogene family	-0.76 ± 0.05	1.83E-05	0.0396
AA250151	-	ESTs, highly similar to auxin-resistance protein axr1 (*A. thaliana*)	-0.98 ± 0.06	1.83E-05	0.0396
AA518585	Osp94	osmotic stress protein 94 kDa	-1.00 ± 0.14	1.98E-05	0.0428
W11216	Rpo2tc1	RNA polymerase II transcriptional coactivator	-0.85 ± 0.08	2.00E-05	0.0432
AA231572		replication-dependent histone H2A.1 gene	-0.53 ± 0.05	2.00E-05	0.0433
AA097223	Bmp1	bone morphogenetic protein 1	-1.27 ± 0.12	2.02E-05	0.0437
W34507	-	RIKEN cDNA 0610041D24 gene	-0.67 ± 0.07	2.16E-05	0.0467
AA168956	Cai	calcium binding protein, intestinal	-0.87 ± 0.07	2.16E-05	0.0467
AA163997	Cd72	CD72 antigen	-0.62 ± 0.06	2.18E-05	0.0471

[a] All values are the mean $\log_2$ ratio ± SE of 9 independent hybridizations, unless otherwise indicated. n less than 9 indicates that a valid estimate of geneexpression was not obtained in one or more of the 9 hybridizations.

[b] Adjusted p values are raw p values adjusted by the step-down Bonferroni method, as described in the Materials and Methods.

Table 3. Genes significantly (raw $p < 0.05$) altered in expression on PND21,105 and 315 in male mice exposed to diethylstilbestrol through gestation and lactation.

Accession	Symbol	Name	PND21[a]	PND105[a]	PND315[a]
AA647753	Gnb1	guanine nucleotide binding protein, beta 1	-0.45 ± 0.12	-0.82 ± 0.11	0.08 ± 0.07
AA755526	-	ESTs, highly similar to ATP synthase delta chain, mitochondrial precursor (*R. norvegicus*)	0.48 ± 0.03	0.26 ± 0.03	-0.03 ± 0.15
AA856411	Pde1c	phosphodiesterase 1C	0.57 ± 0.09	0.37 ± 0.11	-0.20 ± 0.18
AI158037	Creb1	cAMP responsive element binding protein 1	0.64 ± 0.14	0.32 ± 0.07	-0.70 ± 0.36
AI255214	Fin16	fibroblast growth factor inducible 16	-0.44 ± 0.12	-0.28 ± 0.09	-0.45 ± 0.12
W82738	SR-B1	scavenger receptor class B type I	-0.71 ± 0.09	-0.31 ± 0.06	-0.43 ± 0.32
AA030284	Rps19	ribosomal protein S19	0.59 ± 0.09	0.21 ± 0.06	0.18 ± 0.09
AA137833	Supt5h	suppressor of Ty 5 homolog (*S. cerevisiae*)	-0.72 ± 0.18	-0.97 ± 0.06	0.05 ± 0.07
AA245628	-	ESTs, highly similar to NADH-ubiquinone oxidoreductase 18 kd subunit precursor (*B. taurus*)	-0.75 ± 0.13	-0.58 ± 0.19	0.38 ± 0.06
AA199274	Kif2	kinesin heavy chain member 2	-0.66 ± 0.11	-0.77 ± 0.06	-1.16 ± 0.02
AA242192	-	ESTs, weakly similar to histidine decarboxylase (*M.musculus*)	0.68 ± 0.05	0.16 ± 0.06	-0.45 ± 0.23
AA242654	Grn	granulin	0.76 ± 0.05	0.38 ± 0.07	-1.28 ± 0.22
AA543702	Scg3	secretogranin III	0.52 ± 0.05	0.29 ± 0.06	0.06 ± 0.39
AA833322	Unc119h	UNC-119 homolog (*C. elegans*)	0.22 ± 0.06	0.21 ± 0.08	0.19 ± 0.32
AW322547	Fxr1h	fragile X mental retardation gene, autosomal homolog	-0.50 ± 0.08	-0.69 ± 0.05	-0.23 ± 0.08
W91223	-	ESTs, moderately similar to calponin, acidic isoform (*R. norvegicus*)	0.24 ± 0.10	-0.32 ± 0.06	-0.77 ± 0.47
W62227	Stat3	signal transducer and activator of transcription 3	-0.87 ± 0.22	-0.54 ± 0.12	0.25 ± 0.07
AI097696	Trt	translationally regulated transcript (21 kDa)	0.17 ± 0.06	-0.26 ± 0.03	0.18 ± 0.04
AW107708	Cebpa-rs1	CCAAT/enhancer binding protein alpha (C/EBP), related sequence 1	-0.57 ± 0.15	-1.13 ± 0.23	-0.25 ± 0.11
AI894343	Psap	prosaposin	0.51 ± 0.16	0.44 ± 0.10	-0.25 ± 0.23
AI327460	Hba-a1	hemoglobin alpha, adult chain 1	-0.60 ± 0.16	-0.66 ± 0.20	0.15 ± 0.17

Table 3 (cont'd)

AI326932	Cox8a	cytochrome c oxidase, subunit VIIIa	-0.68 ± 0.12	-0.23 ± 0.08	0.00 ± 0.21
W62229	Ube1c	ubiquitin-activating enzyme E1C	0.29 ± 0.08	0.34 ± 0.14	0.30 ± 0.07
AA028309	Crybb1	beta-B1 crystallin	0.64 ± 0.10	0.32 ± 0.11	-0.45 ± 0.27
AA041710	Ercc1	excision repair 1	-0.72 ± 0.14	-0.81 ± 0.16	0.01 ± 0.09
AA068750	Sdf1	stromal cell derived factor 1	0.37 ± 0.31	0.45 ± 0.15	-0.23 ± 0.09
AA204627	Sp3	trans-acting transcription factor 3	0.50 ± 0.05	0.28 ± 0.05	-0.92 ± 0.43
AA208297	Atp1g1	ATPase, Na+/K+ transporting, gamma 1 polypeptide	0.85 ± 0.10	0.50 ± 0.12	-0.94 ± 0.41
AA250170	Slc7a2	solute carrier family 7 member 2	0.21 ± 0.07	0.30 ± 0.08	-1.19 ± 0.53
AA260001	Wee1	wee 1 homolog (*S. pombe*)	-0.47 ± 0.19	-1.17 ± 0.09	-0.95 ± 0.47
AA268438	Lgp85	lysosomal glycoprotein 85kd (CD36 antigen)	-0.63 ± 0.11	-0.34 ± 0.08	-0.47 ± 0.43
AA426866	Stk18	Serine/threonine kinase 18	-0.64 ± 0.11	-0.70 ± 0.10	-0.18 ± 0.13

[a] All values are the mean $\log_2$ ratio ± SE of 5 (for PND21), 9 (on PND105) and 4 (on PND 315) independent hybridizations, unless otherwise indicated. n less than 9 indicates that a valid estimate of gene expression was not obtained in one or more of the 9 hybridizations.

points (4 induced, 8 repressed). Thirty of 32 genes were in agreement with respect to the direction of change on PND21 and 105, but not PND315 (14 induced, 16 repressed). The complete list of all genes on the array and their normalized expression values, ratios and p values can be found in the supplementary data file at http://www.bch.msu.edu/~zacharet/microarray/supplemental/index.html. Raw microarray data may also be obtained by request to the author.

Real-time PCR verifies changes in testicular gene expression

Although reasonable efforts were made to statistically identify genes that showed evidence of differential expression, the small changes and variability in gene expression measurements on the microarray still warrant verification of the results by an alternative and more quantitative method. With this in mind, the statistical analysis of the microarray data was used to prioritize genes for subsequent experimental verification. Additional criteria for selecting genes for verification include the direction and magnitude of change in gene expression, and the probable or known role of the genes in spermatogenesis and testicular function. Therefore, the expression of selected genes in Tables 2 and 3 were verified by real-time PCR. Based on the microarray results, and other results reported in the literature, additional genes were also chosen for analysis by real-time PCR (Table 1).

The intraassay variation for the real-time PCR assay was less than 1 % and the interassay variation was less than 4 % (data not shown). Despite the reproducibility of the assay, there was considerably more variance within

treatment groups as illustrated by the large error bars (Figure 6-7). This indicated large inter-animal variation in gene expression, which is consistent with the variation in response to treatment among litters (Chapter 5). No attempts were made to remove outliers or litters that did not appear to respond to treatment prior to data analysis. Genes selected for verification based on observed changes on the microarray are shown in Figure 6. Additional genes examined are shown in Figure 7. This includes SF-1 and additional genes in the steroidogenic pathway (Figure 7A-D). The nuclear receptors ERα, ERβ, and the AR were also examined due to their importance in testicular development and spermatogenesis (Figure 7E-G). All treatment groups (0, 0.1, 1 and 10 μg/kg) were analyzed by real-time PCR to examine dose-dependent effects.

The testicular receptor 2 (Tr2-11) (a.k.a. TR2, Nr2c1) is an orphan nuclear receptor expressed preferentially in advanced germ cell populations and is implicated in germ cell apoptosis in response to retinoic acid (253,254). The expression of Tr2-11 was significantly increased 1.4-fold on PND105 ($p < 0.01$) (Figure 6A), which was in agreement with the 1.3-fold increase in expression observed on the microarray (supplementary data). There was no difference in the expression of Tr2-11 on PND21 or PND315 (Figure 6A). Inhibin/activin β C (Inhbc) is a member of the TGF-β family of peptides, which are thought to have important paracrine roles in the testis, in addition to their role in stimulating FSH secretion in the pituitary (255). Expression of Inhbc on the microarray was down-regulated on PND21 and 105, and undetectable on PND315 (supplementary data). Real-time PCR revealed

Figure 6. Real-time PCR of genes selected based on microarray analysis. The relative expression level was scaled to make the expression level of the time-matched control sample equal to 100. The results represent the mean ± SE of six animals per treatment group; each from a different litter. NA = not analyzed. * $p < 0.05$; ** $p < 0.01$.

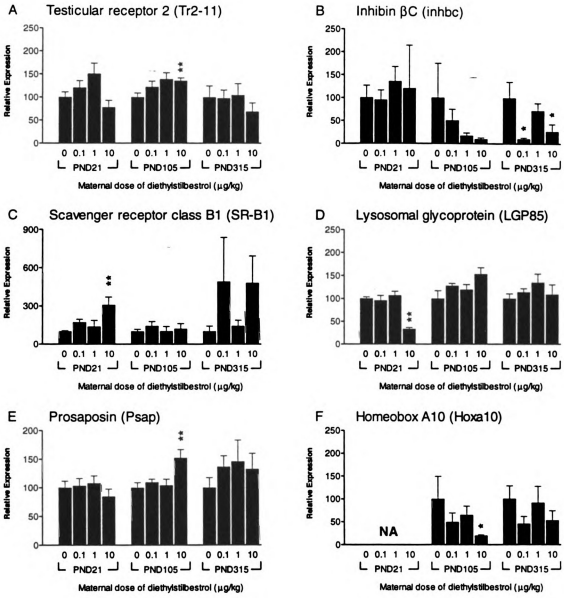
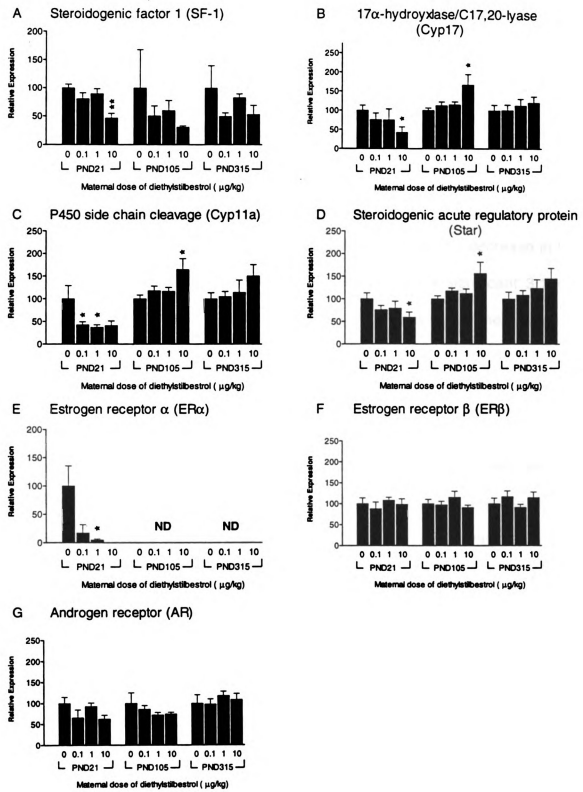

A
Testicular receptor 2 (Tr2-11)
B
Inhibin βC (inhbc)
C
Scavenger receptor class B1 (SR-B1)
D
Lysosomal glycoprotein (LGP85)
E
Prosaposin (Psap)
F
Homeobox A10 (Hoxa10)
Relative Expression
0 0.1 1 10
PND21
PND105
PND315
Maternal dose of diethylstilbestrol (μg/kg)
NA

Figure 7. Real-time PCR of selected genes. The relative expression level was scaled to make the expression level of the time-matched control sample equal to 100. The results represent the mean ± SE of six animals per treatment group; each from a different litter. ND = not detected. Note in (E) that the expression of ERα in all animals of the 10 µg/kg group on PND21 was below the limits of detection. * $p < 0.05$; ** $p < 0.01$.


A
Steroidogenic factor 1 (SF-1)
B
17α-hydroxylase/C17,20-lyase (Cyp17)
C
P450 side chain cleavage (Cyp11a)
D
Steroidogenic acute regulatory protein (Star)
E
Estrogen receptor α (ERα)
ND
ND
F
Estrogen receptor β (ERβ)
G
Androgen receptor (AR)
Relative Expression
0 0.1 1 10
PND21
PND105
PND315
Maternal dose of diethylstilbestrol (μg/kg)

that expression of Inhbc was also decreased 5.8- and 10.0-fold in the 1 and 10 µg/kg DES group, respectively, on PND105, although the results were not significant (Figure 6B). The expression of Inhbc on PND315 was significantly decreased 9.3- and 3.7-fold ($p < 0.05$) in the 0.1 and 10 µg/kg DES groups, respectively (Figure 6B). There was no change in Inhbc expression on PND21. The scavenger receptor class B1 (SR-B1) is a transmembrane protein that mediates selective cholesterol uptake by steroidogenic tissues, including the Leydig cells (256). Although the microarray analysis indicated a decrease in the expression of SR-B1 (Table 3), real-time PCR indicated a significant 3.1-fold increase in SR-B1 expression on PND21 ($p < 0.01$) (Figure 6C). There were no significant changes in SR-B1 expression on PND105 or 315; however, SR-B1 expression was increased approximately 4.8-fold on PND315 in both the 0.1 µg/kg group ($p > 0.33$) and the 10 µg/kg group ($p = 0.0547$) (Figure 6C). The major lysosomal membrane glycoprotein LGP85 (a.k.a. CD36 antigen (thrombospondin receptor)-like 2) is thought to play a constitutive role in the lysosomal endocytotic pathway (257). Its expression was significantly decreased 3.0-fold in the 10 µg/kg group on PND21 ($p < 0.01$). By contrast to the microarray results (Table 3), expression of LGP85 was not affected on PND105 and 315 (Figure 6D). Prosaposin (Psap, a.k.a. Sgp-1) is a sphingolipid activator protein synthesized by the Sertoli cells and thought to be involved in phagocytosis of residual bodies (258,259). Expression of Psap was significantly increased 1.5-fold on PND105 ($p < 0.05$) in the 10 µg/kg group (Figure 6E), which is consistent with the increase observed on the microarray (Table 3). In

contrast to the microarray analysis, there was no change in Psap expression on PND21 or PND315. The inconsistencies between the microarray data and the real-time PCR data for LGP85 and Psap probably reflect false positives, as those genes were selected from Table 3 where raw p values were used in the analysis. Abdominal B-related Hoxa10 gene is involved in normal development of the male reproductive tract, including testicular descent and development of accessory sex organs (260,261). Microarray analysis indicated that Hoxa10 expression was down-regulated on PND105, but unaltered on PND315 (Table 2). In agreement with this observation was a significant 5-fold decrease in expression on PND105 ($p < 0.05$), but not PND315 (Figure 6F). Due to limiting amounts of RNA, expression of Hoxa10 was not analyzed on PND21.

The expression of SF-1 (a.k.a. Ad4BP, Nr5a1) was examined by real-time PCR due to the role of SF-1 in regulating expression of SR-B1 (262), the presence of SF-1 response elements in the Tr2-11 (Accession: U96095) and Hoxa10 (Accession: AF246720) promoters (unpublished observations), as well as the reported down-regulation of SF-1 following *in utero* exposure to DES (225). The expression of three SF-1 target genes in the steroidogenic pathway were also examined; 17α-hydroxylase/C17,20-lyase (Cyp17), cytochrome P450 side chain cleavage (Cyp11a), and steroidogenic acute regulatory protein (Star) (Figure 7B-D). Expression of SF-1 was significantly decreased 2.1-fold on PND21 in the 10 µg/kg group ($p < 0.01$) (Figure 7A). Expression of SF-1 on PND105 and 315 in the 10 µg/kg group was also decreased 3.5- and 1.9-fold, respectively; however, the results were not significant (Figure 7A). Consistent

with the decrease in SF-1, the expression of Cyp17, Cyp11a and Star were all significantly decreased 2.3-, 2.4-, and 1.7-fold, respectively, on PND21 ($p < 0.05$) (Figure 7B-D). However, the expression of each of these genes was unexpectedly increased 1.6- to 1.7-fold on PND105 in the 10 µg/kg group ($p < 0.05$). There was no change in the expression of these genes on PND315.

ERα expression was significantly decreased on PND21 in the 1 µg/kg group ($p < 0.05$) (Figure 7E). Expression of ERα was below detectable levels in the 10 µg/kg group, indicating that expression was also significantly decreased in this treatment group. Expression of ERα was also below detectable levels in all treatment groups on PND105 and 315 (Figure 7E). Attempts were made to repeat the RT-PCR with more RNA and/or more input cDNA, but expression could not be reproducibly detected to perform statistical analysis. Expression of ERβ and AR were not altered by DES treatment (Figure 7F-G). Other genes analyzed by real time PCR that were not significantly altered by treatment include Zona pellucida 3 receptor (Zpr3), Xlr-related, meiosis regulated (Xmr), CD36 antigen, Secreted acidic cysteine rich glycoprotein (Sparc), and Bcl-associated death promoter (Bad).

DISCUSSION

Numerous studies have established that *in utero* or neonatal exposure to DES causes long-term adverse effects on testicular development and sperm quality (81,82,89,91,192,194,240). Evidence suggests that exposure to DES causes direct effects on the somatic cells of the testis by disrupting Sertoli cell maturation (89) and steroidogenesis in the Leydig cells (263-265), and indirect effects by disruption of the hypothalamic-pituitary-gonadal axis (15,91,192,197). The expression of the ER in germ cells (ERβ) and Leydig cells (ERα) (30) are also consist with ER-mediated effects on these cells. The expression of the ER in the Sertoli cells of mice has not been demonstrated, although immunodetection of ERβ in the Sertoli cells of the rat has been documented (33,35). The molecular mechanisms responsible for the effects of DES on the testis and spermatogenesis remain elusive, and few estrogen responsive genes have been described in the testis. By contrast, a large number of genes in the uterus have been shown to be altered by *in utero* or neonatal exposure to DES (211,241-246). Studies by Majdic *et al.* (92,225) have demonstrated that *in utero* exposure to DES alters expression of SF-1 and Cyp17 in the fetal Leydig cells of rats, thus suggesting that the effects of DES on spermatogenesis are at least in part a result of inhibition of steroidogenesis. Neonatal exposure to high doses of DES also affects the expression of testicular inhibin α, Psap, and AR in prepubertal rats (89,208). The results of the present study confirm the down-regulation of SF-1 and Cyp17, and through the use of cDNA microarrays, add

support to the hypothesis that the adverse effects of DES are a result of changes in testicular gene expression. Transient and latent alterations in the expression of genes involved in estrogen signaling (ERα), steroidogenesis (SF-1, Star, Cyp17, Cyp11a, SR-B1), lysosomal function (LGP85, Psap), and regulation of testicular development (Tr2-11, Inhbc, Hoxa10) were also observed, even long after the cessation of DES exposure. Decreased expression of these genes may contribute, together or in part, to the decrease in testicular growth, epididymal sperm count and sperm fertilizing ability *in vitro* as a result of 1) decreased testicular synthesis of C19 steroids, or 2) suppression of ERα signaling, both of which are essential for fertility (266). A third mechanism may involve altered function of the supporting somatic cells of the testis leading to malfunction of spermatogenesis and/or alterations in the responsiveness of germ cells to maturation cues, which are necessary for the acquisition of fertilizing ability. Possible effects on gene expression in the efferent ducts and/or epididymis also can not be ruled out as they play crucial roles in the maturation of spermatozoa.

cDNA microarrays and statistical testing identify differentially expressed genes in the testis of DES exposed mice

This study has demonstrated the utility of cDNA microarrays for discovering previously unidentified molecular alterations following gestational and lactational exposure to DES. Despite the reproducibility of the microarray assay and the relatively low biological variation between control animals, the results of real-time PCR illustrate the importance of verifying changes in gene expression in order to minimize the number of false positive claims. However, the use of

statistical testing was clearly advantageous in reducing the number of false negatives, as bona fide changes in gene expression as low as 1.5-fold (Psap) could be identified. This illustrates the limitation of using a commonly applied and arbitrary 2-fold cutoff for defining significant differences in gene expressison. Statistical testing was crucial, since the long-term effects on testicular gene expression following developmental exposure are likely to be highly variable between animals, thus limiting our ability to detect small but real changes in gene expression. The changes in gene expression are also likely to be confined to discrete cell types, which may result in large changes being diluted by the heterogeneous cellularity of the testis. Considerable research has been invested in developing and exploring appropriate methodologies for statistically analyzing microarray data and controlling the family wise type 1 error rate (267-270). Mixed model analysis of variance models are also currently being explored to identify additional candidate genes and to compare the analytical methodologies (P. Saama et al., unpublished data). Although emerging experimental designs and statistical methods may prove superior in power and sensitivity, the use of paired t-tests and p values for prioritizing differentially expressed genes has the advantages of well established experimental and theoretical support, and simplicity of implementation and interpretation. Pre-selecting treated animals for microarray analysis based on positive physiological responses, rather than including all available animals, may have also reduced the between-litter variation in gene expression response and increased the success of identifying responsive genes. Nonetheless, this approach has proven to be successful for

identifying novel molecular targets that lead to new hypotheses regarding potential mechanism(s) of action of DES on testicular development and sperm quality.

DES exposure represses mRNA expression of ERα, but not ERβ or AR

Gene knockout technology has established an essential role for ERα in the somatic cells of the testis and the efferent ducts for normal fertility in the mouse (37,40,271). ERα in the efferent ducts regulates epithelial ion transporters involved in fluid reabsorption, which is required for concentrating sperm in the head of the epididymis (39). By contrast, the role of ERα in the somatic cells of the testis is unknown. Many lines of evidence point to a role for ERα in steroidogenesis and Leydig cell growth and regeneration. For example; (i) steroidogenesis in the Leydig cells is directly inhibited by E2 treatment *in vivo* and *in vitro* (265,272,273), (ii) *in utero* treatment of rats leads to disruption of Leydig cell development in mature animals (274,275), and (iii) estrogen treatment blocks the regeneration of new Leydig cells in ethane-dimethylsulfonate-treated adult rats (276). Histological examination of testis sections from DES-exposed mice revealed no treatment related effects (Chapter 5), indicating that Leydig cells were morphologically normal. This is in agreement with the results of Majdic *et al.* (92) where *in utero* exposure to even higher doses of DES did not alter testicular histology or the number of Leydig cells in fetal rats. Therefore, the decrease in ERα expression may have functional consequences on Leydig cell responsiveness to LH and testosterone production. Although this study did not measure testosterone levels, other studies have demonstrated decreases in

testosterone and 17α-hydroxylase activity in fetal rats exposed to E2 or DES *in utero* (92,204). E2 and DES also inhibit LH-induced testosterone production *in vivo* and *in vitro* (277-279). The decreased expression of Cyp17 in the testis following *in utero* exposure (11.5 to 15.5 p.c) to DES (92,225), and the decreased expression of Cyp17, Cyp11a and Star following *in utero* and lactational exposure to DES in the present study (Figure 7), suggest a possible mechanism for the decrease in testosterone production. The decrease in testosterone production may also be a result of an estrogen-induced reduction in the number of Leydig cell LH-binding sites (280), or by competitive inhibition of 17α-hydroxylase activity or inhibition of cAMP formation (279,281,282). Non-ERα mediated effects on steroidogenesis are also possible as the antiestrogen tamoxifen does not block the inhibitory effects of estrogens on testosterone production, nor does tamoxifen-induced down-regulation of ERα prior to E2 treatment (278). The exact role of ERα, if any, in regulating enzymes in the steroidogenic pathway is unclear. One report has demonstrated a role for ERα in synergizing with SF-1 to regulate gonadotrope-specific expression of the gonadotropin IIβ subunit gene in salmon (283). However, this interaction in steroidogenic cells has not been shown. The decrease in ERα expression may have alternative consequences on germ cell development and/or Sertoli cell maturation (40). Attempts to discover estrogen-regulated genes in the testis have been unsuccessful (284). However, the reduced sperm count and *in vitro* sperm fertilizing ability in both the DES-exposed mice (26) and ERα knockout

mice (39) suggest a causal link between down-regulation of ERα and the adverse effects on sperm quality.

The mode by which gestational and lactational exposure to DES alters expression of ERα is unclear. Previous studies have shown that treatment of neonatal rats with supraphysiological doses of DES imprints ERα, ERβ and AR in the testis (285). Expression of ERα and AR mRNA was decreased in neonatal and prepubertal testes, whereas ERβ mRNA was increased over the same time period. Furthermore, this effect was not mimicked by GnRH antagonist treatment, indicating that the effect of DES on the testis may be independent of its effect on the pituitary. While ERα expression was decreased on PND21 in the current study, expression of ERβ and AR were not altered (Figure 7). This argues that receptor expression was not imprinted as previously observed, and probably involves alternate mechanisms. Differences in the timing, dose or route of exposure may also explain the distinct expression patterns induced by DES. Although there was a decrease in the number of Sertoli cells, ERα is not known to be expressed in these cells but rather is confined to the Leydig cells (30). Therefore, the decrease in ERα expression is not likely due to changes in the number of Sertoli cells. The effect of exogenous estrogen treatment on ER expression appears to be cell-, dose-, and age-dependent (286-290), and it remains to be determined what is regulating both ERα or ERβ expression in the developing testis.

DES exposure represses mRNA expression of genes required for steroidogenesis

The decreased expression of SF-1 and other enzymes in the steroidogenic pathway (i.e. Cyp17, Cyp11a, Star) confirm the previously reported decrease in SF-1 and Cyp17 expression following *in utero* exposure to DES (92,225). These results support the hypothesis that the effects of DES on testicular development and sperm quality are, at least in part, a result of a suppression in steroidogenesis. Basal and cAMP-induced expression of Cyp17, Cyp11a and Star are positively regulated by SF-1 (291-293). Therefore, the decreased expression of Cyp17, Cyp11 and Star is consistent with the decrease in SF-1 mRNA (Figure 7), although the involvement of other factors cannot be disregarded. For example, neonatal treatment of female rats with estradiol benzoate decreases ERα, Star and Cyp11a expression in the ovary, while SF-1 expression is unaffected (294). This suggests that expression of ERα may be required for full expression of Star and Cyp11a in the neonatal ovary, possibly though direct interactions with SF-1 or by cooperativity via their respective response elements (283). ERα may also regulate the expression of other factors necessary for SF-1 activity. Therefore, it is tempting to speculate that loss of ERα expression in the perinatal testis may be required for basal or hormone-induced expression of Star or Cyp11a. Despite the evidence described, other studies have shown that expression of SF-1 is not affected or is up-regulated in the testis following estrogen exposure *in utero* (78,197). These discrepancies may be due to differences in dose or timing of exposure, and possibly species.

Nonetheless, the data suggests a mechanistic link between estrogen signaling and SF-1 activity in the regulation of steroidogenesis.

One possible mechanism that may explain the decreased expression of SF-1 responsive genes is cross-talk between ERα, estrogen-related receptor (ERR) α and SF-1 on SF-1 response elements (295). The finding that DES can inhibit the constitutive transcriptional activity of ERRα (187,223), which is expressed in the developing testis (224), raises the possibility that DES may disrupt SF-1 dependent transactivation via competitive interference with SF-1 response elements by liganded ERα and/or ERRα. Although SF-1 functions as a monomer, numerous studies have shown that SF-1 can cooperate with other proteins to enhance gene expression, including SRC-1 (296), C/EBPβ (297), ERα (283), RAR (298), Egr-1 (299), Wnt-1 and Dax-1 (300), SOX-9 (301), Sp1 (302), CREB (303), and the AR (304). These interactions likely account for the cell- and promoter-specific effects of SF-1 on gene expression. Altered expression or activity of other interacting partners may explain the decrease in Cyp17, Cyp11a or Star mRNA observed in this study. For example, functional interactions between SF-1 and CREB (cAMP response element binding protein) are important for basal and cAMP-induced expression of aromatase in Leydig cells (303). Although not confirmed, the putative decrease in CREB expression observed on the microarray (Table 3) may result in a decrease in the expression of aromatase and possibly other steroidogenic enzymes in the Leydig cells. In contrast to this hypothesis, the expression of the SF-1 target gene SR-B1 was found to be up-regulated on PND21 (Figure 6C). SF-1 mediates cAMP-

inducibility of SR-B1 synergistically with sterol regulatory element-binding protein-1a (305,306). The increase in SR-B1 facilitates an increase in the cellular uptake of cholesterol substrates for steroidogenesis, which may represent a mechanism to compensate for the decrease in steroid synthesis. How SR-B1 is up-regulated when SF-1 expression is decreased is unknown, but may reflect other distinct regulatory mechanisms which compensate for the lack of SF-1-mediated transactivation. These mechanisms may also explain the coordinate up-regulation of Cyp17, Cyp11a and Star in the 10 µg/kg group on PND105 (Figure 7) following their down-regulation on PND21. Whether up-regulation of these enzymes result in increased testosterone production, or contribute to the decrease in sperm fertilizing ability is unknown, and it is premature to speculate in the absence of protein or enzyme activity data.

Novel targets of early DES exposure suggest multiple mechanisms of DES-induced reproductive toxicity

The expression of Tr2-11 was increased on PND105, and it appeared to increase with increasing doses of DES (Figure 6A). Postnatal expression of Tr2-11 coincides with the onset of meiosis and is present mainly in advanced germ cell populations. The expression of Tr2-11 is repressed by p53 and androgens, and induced by retinoic acid (RA) (254,307,308). Due to its role in repression of basal and RA-induced gene expression, as well as apoptosis (254), the increase in Tr2-11 may play a role in suppressing germ cell differentiation and/or increasing the susceptibility of germ cells to undergo apoptosis.

There was a latent and significant decrease in Inhbc expression on PND315 (Figure 6B). This effect was more pronounced on PND105, although the results were not significant due to the large variation in the control group (Figure 6B). The function of this inhibin βC subunit is not known, and has not been shown to dimerize like the βA and βB subunits (255). Therefore, the function of Inhbc in the testis is unknown, and more functional information for Inhbc is needed to interpret these results. This example highlights the importance of using a microarray with well characterized genes in order to generate reasonable hypotheses, and points to the limitations of ESTs in the context of certain experimental designs.

DES caused a transient down-regulation in testicular LGP85 mRNA on PND21, but not on PND105 or 315 (Figure 6D). LGP85 is a major lysosomal membrane glycoprotein that appears to be expressed constitutively in all mouse tissues (309), although the function of this protein is unknown. The decrease in LGP85 expression may reflect a general decrease in lysosomal biogenesis, which could have functional consequences in cellular autophagy by Sertoli cells, an important process for the phagocytosis of residual bodies. It is interesting to note that lysosomes and other organelles of the endocytotic pathway are less developed in the nonciliated cells of the efferent duct in ERα knockout mice (310). If ERα also plays a role in regulating components of the endocytotic pathway in the testis, the decrease in ERα expression in the testis of DES-exposed mice on PND21 could explain the decrease in LGP85 mRNA at this time point.

Psap, or sulfated glycoprotein-1 (SGP-1), was significantly increased in expression on PND105, but not on PND21 or 315 (Figure 6E). Psap is synthesized in Sertoli cells and serves at least two functions. It is targeted to the lysosomes where it plays a role in the hydrolysis of membrane glycolipids found in phagocytosed residual bodies (258). Psap is also secreted into the lumen of the seminiferous tubules where it binds to late spermatids, and may play a role in the transfer of glycolipids from Sertoli cells to late spermatids. Previous studies have demonstrated that Psap expression in the neonatal rat is a marker for Sertoli cell maturation, and neonatal exposure to DES diminishes its expression pattern (89). This is consistent with a decrease in the maturation and number of Sertoli cells. Considering the long-term decrease in Sertoli cell number, a reduction in Psap expression would be expected rather than the increase observed in this study. If adult testicular Psap is regulated by pituitary factors (311), then the increase in Psap mRNA on PND105 may reflect an increase in gonadotropin secretion. This scenario may also explain the increased expression of Cyp17, Cyp11a and Star (Figure 7).

Developmental exposure to DES causes a latent decrease in the expression of Hoxa10 in the adult testis (Figure 6F). The role of Hoxa10 in the testis is unknown, although Hoxa10 is known to play a role in segmental patterning of axial structures, including the Wolffian duct and urogenital sinus (261). Homozygous knockouts of Hoxa10 manifest bilateral cryptorchidism due to developmental abnormalities of the gubernaculum, which ultimately results in defects in spermatogenesis and sterility (260,312). Altered fetal expression of

Hoxa10 in the testis may play a role in DES-induced cryptorchidism previously observed (74,80,172). It is interesting to note that Hoxa10 expression is disrupted in the uterus of adult Wnt-7a knockout mice (313), and that Wnt-7a expression is down-regulated in fetal mice exposed *in utero* to DES (244). The link between DES-induced down-regulation of Wnt-7a and its rōle in maintaining adult expression of Hoxa10 leads to the hypothesis that developmental exposure to DES may cause reproductive abnormalities in gubernaculum and uterine development as a result of disruption of the Wnt-7a/Hoxa10 pathway. Estrogen response element half sites have also been reported in the mouse Hoxa10 promoter, thus raising speculation that disruption of estrogen signaling may persist to adulthood, thus causing a decrease in Hoxa10 expression (314).

With the use of cDNA microarrays and real-time PCR, previously unidentified changes in gene expression have been identified and associated with adverse effects on testicular development and sperm quality following gestational and lactational exposure to DES. This includes genes involved in Leydig cell function, steroidogenesis, lysosomal function and testicular development. Establishing effects at the physiological, tissue, and cellular level, and associating them with changes at the molecular level allows us to explore and generate working hypotheses explaining the molecular mechanisms of developmental reproductive toxicants. Similar studies assessing the effect of other potent (ethynyl estradiol) and weak estrogen agonists (genistein) on testicular development, sperm quality and testicular gene expression are ongoing. Comparison of the effects on testicular gene expression with diverse

estrogen agonists will allow us to 1) establish biomarkers of effect for estrogens on testicular development and sperm quality, 2) determine agonist-specific effects on the testis, and 3) strengthen or dispute the hypotheses regarding the mechanism(s) of action of EEDs on male reproductive health.

CHAPTER 7

GP3: GENEPIX POST-PROCESSING PROGRAM FOR AUTOMATED ANALYSIS OF RAW MICROARRAY DATA[5]

ABSTRACT

Here we describe an automated and customizable program to correct, filter and normalize raw microarray data captured using GenePix, a commonly used microarray image analysis application. Files can be processed individually or in batch mode for increased throughput. User defined inputs specify the stringency of data filtering and the method and conditions of normalization. The output includes gene summaries for replicate spots and descriptive statistics for each experiment. The source code (Perl) can also be adapted to handle raw data output from other image analysis applications. Availability: http://bch.msu.edu/~zacharet/microarray/GP3.html.

[5] Published in Fielden M.R., Halgren R.G., Dere E., Zacharewski T.R. (2002) GP3: GenePix post-processing program for automated analysis of raw microarray data. Bioinformatics. In press.

INTRODUCTION

Image analysis of two-color fluorescent cDNA microarray produce a large number of raw data points describing the spot fluor (e.g. Cy5 or Cy3) intensity, background fluor intensity, and a variety of other spot quality measurements. Typically, the raw spot intensity values for one fluor are corrected for background signal and then compared to the corrected spot intensity value of the other fluor to generate a ratio. This ratio represents the relative difference in gene expression between the two samples co-hybridized on the microarray (e.g. control and test cDNA). However, systematic and experimental biases can exist between the two fluor-labeled cDNA populations, resulting in inaccurate quantitation of relative differences in gene expression. The error is often associated with i) differences in the efficiency of incorporation of fluor-labeled nucleotides into the cDNAs by reverse transcriptases, ii) differences in the stability and fluorescence emission characteristics of the fluors, and iii) differences in RNA loading, quality and sample handling. As a result, signal intensity values for each fluor are often normalized, or transformed, by a correction factor. This mathematical correction attempts to remove systematic and experimental biases in fluor characteristics so that accurate ratios can be calculated (315,316). Furthermore, failure to remove spots below threshold levels (i.e. no expression) or at saturating levels can lead to invalid or undefined ratios.

Data filtering, correction, and normalization of raw data to produce valid ratios are often performed manually using spreadsheets, which can be time consuming and prone to error. Summarizing the results from experiments and tracking replicate spots and errors can also be very time consuming. In order to automate and increase the throughput of processing raw microarray data, while simultaneously minimizing human intervention, a script was developed to automatically process raw microarray data from GenePix image analysis software (Axon Instruments, Union City, CA).

ALGORITHM

The algorithm uses a threshold to define detectable expression in order to filter those spots that are considered below the limits of detection and not appreciably different from background values. A spot is considered below the limit of detection if

$$S_{ij} < B_{ij} + x\sigma_{Bij} \qquad [1]$$

where S_{ij} is the median spot signal intensity for gene i (i = 1,...,n genes on the array) in channel j (j = 1 or 2), B_{ij} is the median background spot intensity for gene i in channel j, x is a user defined threshold (default = 3), and σ_{Bij} is the standard deviation of B_{ij}. If the spot is flagged in one channel but not the other, S can be set to a user-defined baseline value to avoid undefined ratios (default raises S to the threshold level for that gene according to Equation 1). A flag is set in the output file to indicate that the calculated ratio may be inaccurate as one channel was below the limits of detection. If the spot is flagged in both channels, the gene is removed from any further analysis and no valid ratio is calculated. Spots are also flagged if S is saturated (i.e. S = 65536 for GenePix) in either channel. This indicates that the calculated ratio may be inaccurate since the true value for S is unknown. If S is saturated in both channels, the gene is removed from any further analysis and a valid ratio is not calculated. Spots are corrected for background signal to produce a corrected spot signal intensity (S') according to Equation 2.

$$S'_{ij} = S_{ij} - B_{ij} \qquad [2]$$

Corrected spot signal intensities (S') are normalized by one of two linear normalization methods as defined by the user input. This step scales the distribution of log ratios closer to a mean of zero, such that the distributions of intensities are equivalent and comparisons between channels are more accurate. Intensity values are normalized in log space in order to make normalization additive and to make the variation in intensity less dependent on absolute magnitude. The two normalization options are termed 1) z-score normalization and 2) global normalization.

The z-score normalization is a linear transformation applied to the $\log_2 S'$ values so that the distribution of z-score normalized values has zero mean and unit variance for that channel. This is done by scaling the $\log_2$ signal intensity ($\log_2 S'$) of each spot on the array by subtracting the mean of all (n) $\log_2 S'$, or a subset ($n - m$) of $\log_2 S'$, and dividing by its standard deviation (Equations 3 and 4). The scaled result is inverse transformed and termed the normalized signal intensity (N_{ij})

$$N_{ij} = 2^{(\log_2 (S'_{ij}) - X_j)/\sigma(X_j)} \qquad [3]$$

where X_j is

$$X_j = \frac{\sum_{i=m}^{n} \log_2 (S'_{ij})}{n-m} \qquad [4]$$

and $\sigma(X_j)$ is the standard deviation over the same set of spots used to calculate X_j.

A subset of $\log_2 S'$ may be appropriate when values at either end of the distribution may inappropriately bias the scaling factor. This may occur when comparing two very distinct tissue types with divergent expression profiles. Only spots that are not flagged are included in the calculation of X and $\sigma(X)$. To exclude outliers at either end of the distribution of $\log_2 S'$, a trimmed X (i.e. a subset of $\log_2 S'$) and its standard deviation can be calculated as defined by the user input. By default, a 90 % trimmed mean of valid spots is calculated for X, such that 5% of the values at either end of the distribution are excluded from X. This assumes approximately 90 % of the genes on an array will be unchanged by treatment. The value chosen will depend on the expected degree of variation between the two samples being compared. More divergent samples may require a smaller subset (i.e. 50 %).

The global normalization method scales the $\log_2$ signal intensity ($\log_2 S'$) of each spot on the array by subtracting the mean of all (n) $\log_2 S'$, or a subset ($n - m$) of $\log_2 S'$ (Equation 5).

$$N_{ij} = 2^{\log_2(S'_{ij}) - X_j} \qquad [5]$$

Regardless of the method used to normalize, both the normalized ratios (R) and $\log_2$ transformed ratios (R') of channel 1 and channel 2 are calculated for gene i according to

$$R_i = \frac{N_{i1}}{N_{i2}} \qquad [6]$$

$$R'_i = \log_2 R_i \qquad [7]$$

The results of data filtering, correction, and normalization are appended to the raw data in a comma separated values (CSV) file to facilitate graphing and visualization of the results and to preserve the original raw numbers. The CSV files can be visualized opened in a spreadsheet program, such as Microsoft Excel. The file includes flag fields for spots that do not pass the threshold criteria in channel 1 or 2, as defined by Equation 1, as well as a flag for spots that were saturated in channel 1 or 2. This is used to judge the accuracy of the ratio measurements. Log_2S', N, R, R' are recorded for all valid spots on the microarray. The geometric mean of the signal intensity (*S*) in both channels is also calculated (denoted G) and appended to the results, in addition to log_2G and its percentage of maximum (100 x G/65353). This is used primarily for graphing and estimating expression levels.

A second CSV file is produced to summarize the normalized signal intensity values (arithmetic mean, standard deviation, and coefficient of variation) for replicate spots on the array. The average signal intensity data (i.e. average of G, log_2G and its percentage of maximum across replicates) are also included in the summary file.

A descriptive file is produced for each microarray to summarize the results of the experiment. This includes the header information from the GenePix results

file and the user-defined parameters selected for the analysis. Descriptive statistics and a summary of the experimental results include a summary of the flags, normalization factors, correlation between channels, average spot and background signal intensities, and the distribution and percent distribution of valid ratios.

IMPLEMENTATION

The Perl-based script is available for download at http://bch.msu.edu/~zacharet/microarray/GP3.html. The program was written in Perl 5.6.0 and requires the external Perl module Statistics::Descriptive v. 2.3 (authored by Colin Kuskie). The module is publicly available at www.CPAN.org. Although the program can currently accommodate only GenePix result files, the available source code can be easily modified to accommodate a variety of file formats. The script can be executed to process single files or entire directories for increased throughput. Documentation for use can be found at http://bch.msu.edu/~zacharet/microarray/GP3.html.

Due to the wide use of GenePix as a microarray image analysis application, as well as the necessity for a simple, intuitive and effective means of processing raw microarray data in a fast and customizable manner, we expect researchers using microarrays to find this script of great utility. It is also expected that researchers using other image analysis applications will modify the existing source code to accommodate other output file formats.

CHAPTER 8

CONCLUSIONS AND FUTURE PERSPECTIVES

Conclusions

The effects of DES on testicular development and sperm quality do not predict the effects of weaker estrogens, such as Aroclor 1242. Differences in adsorption, disposition, metabolism, excretion, potency, duration of exposure and dose may contribute to the unique effects induced by EEDs, in addition to other inherent biological activities of the chemical. For both DES and Aroclor 1242, alterations in testis size and epididymal sperm count and motility did not predict alterations in sperm fertilizing ability *in vitro*. These results indicate that many current testing procedures for male reproductive toxicity are not predictive or sensitive enough to detect chemicals with endocrine disrupting activity. Furthermore, effects observed in prepubertal and early adult mice are not predictive of effects at later stages of life. The effects of EEDs can persist beyond early adulthood, and may develop or become more pronounced with age. With the use of cDNA microarrays and real-time PCR, changes in gene expression have been quantitated and associated with adverse effects on testicular development and sperm quality following gestational and lactational exposure to DES. This includes genes involved in estrogen signaling, Leydig cell function, steroidogenesis, lysosomal function and testicular development. Finally, establishing effects at the physiological, tissue, and cellular level, and

associating them with changes at the molecular level allows us to explore and generate working hypotheses that may explain the molecular mechanisms of developmental reproductive toxicants, and identify critical pathways involved in spermatogenesis.

Future perspectives

cDNA microarrays represent a major technological advancement that facilitates the simultaneous analysis of the expression level of thousands of genes in multiple samples. This technology has facilitated the identification of a number of previously unidentified molecular targets that are altered in expression in the testis following gestational and lactational exposure to DES. These findings suggest a number of hypotheses that may explain the effects of DES, and possibly other EEDs, on testicular development and sperm quality.

Future experiments would be required to further confirm the functional importance of the currently identified alterations in gene expression. This would include determining the exact periods of development when the changes in expression occurred, and how long the changes persisted. Immunohistochemistry could also be used to determine the localization of protein expression in the testis, and to confirm whether changes in mRNA were accompanied by changes in protein level. Functional assays may also be required to determine if the changes in protein level were accompanied by changes in protein activity.

Concurrent studies examining the effects of gestational and lactational exposure to other EEDs, including the weakly estrogenic isoflavone genistein (GEN), and the potent synthetic estrogen ethynyl estradiol (EE), will indicate whether the effects of DES at the molecular, cellular and tissue level are similar to GEN or EE, or unique to DES. Target genes induced or repressed by DES, GEN and EE will substantiate the hypothesis that the changes in gene expression are a result of ER-mediated events. These observations will support the use of these target genes as biomarkers of exposure to EEDs.

Finally, to determine if these alterations in gene expression are necessary or sufficient to cause alterations in testicular development and sperm quality, more detailed studies are required. This may include the use of gene knockout technology. The use of anti-sense oligonucleotide inhibition of gene expression provides an effective means to inhibit fetal or adult expression of specific genes in a dose- and time-dependent manner, and to determine developmental inhibition of gene expression by anti-sense technology recapitulates the phenotype induced by DES exposure. Potentially interesting target genes include ERα, SF-1, LGP85, or Hoxa10. Functional analysis of gene regulatory mechanisms will also be required to understand how these genes are regulated at the transcriptional level, and to understand how DES interferes with this regulation.

APPENDIX A

SAS™ SCRIPT FOR STATISTICAL ANALYSIS OF MICROARRAY GENE EXPRESSION DATA

```
# Import data must be arranged in columns containing normalized expression
# values for each animal. E.g. treated animals are labeled 'PND21_Des_1' to
# 'PND21_Des_5' and control animals are 'PND21_Ctl_1' to 'PND21_Ctl_5'. The
# key is TRZID, a unique identifier for each gene. This script will invoke a paired
# t-test to analyze gene expression differences on PND21 using the
# supplementary data set published on the web (See Appendix B), as described
# in Chapter 6 Materials and Methods.

PROC IMPORT OUT= MARK.DATA
        DATAFILE= "C:\Data\DES_Normalized_Expression_Data_all.xls"
        DBMS=EXCEL2000 REPLACE;
        GETNAMES=YES;
RUN;

TITLE 'paired t-test';
DATA Test;
        set MARK.DATA;
        treated=PND21_Des_1; control=PND21_Ctl_1; output;
        treated=PND21_Des_2; control=PND21_Ctl_1; output;
        treated=PND21_Des_3; control=PND21_Ctl_1; output;
        treated=PND21_Des_4; control=PND21_Ctl_1; output;
        treated=PND21_Des_5; control=PND21_Ctl_1; output;
        keep TRZID treated control;
RUN;

PROC TTEST data=Test;
        by TRZID;
        paired treated*control;
        ods listing exclude ttests; ods output ttests=ttests;
RUN;

DATA ttests;
        set ttests;
        rename probt=raw_p;
RUN;
```

```
PROC MULTTEST pdata=ttests stepbon pvals out=adjustedps;
RUN;

PROC SORT data=adjustedps;
        by TRZID;
RUN;

PROC SORT data=ttests;
        by TRZID;
RUN;

DATA results (drop=variable);
        merge adjustedps ttests;
        by TRZID;
RUN;

PROC EXPORT DATA=WORK.RESULTS
        OUTFILE= "C:\Data\PND21_Expression_Results.xls"
        DBMS=EXCEL2000 REPLACE;
RUN;
```

APPENDIX B

SUPPLEMENTARY MICROARRAY DATA

The complete list of all genes on the microarray (Clone_id, GenBank Accession, Unigene Cluster ID, Gene Name, Gene Symbol, MGI, LocusLink) and their normalized expression values for each animal, ratios of fold-induction (SD,N,SE), t-statistics, and raw and adjusted p values can be found in the supplementary data file at http://www.bch.msu.edu/~zacharet/microarray/supplemental/index.html.

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