Gene pathways targeted during phthalate-induced testicular dysgenesis

Kevin W. Gaido, Ph.D.
Hamner Institutes for Health Sciences
Research Triangle Park, NC USA
Presentation Outline

- Phthalate profile study
- Time course
- Promoter analysis
- Species comparison
Public Health Issue

- Reported increases in the incidence of cryptorchidism, hypospadias, prostate and testicular cancer, and reduced fertility.

- Testicular dysgenesis syndrome (TDS)
  - Disruption of male reproductive tract development during fetal life by genetic or environmental factors.
Laboratory Observations

- Exposure of fetal male rats to antiandrogens leads to reproductive disorders that are similar to those of concern in the human male population.
  - Cryptorchidism, reproductive tract defects, reduced fertility, cancer
Environmental Antiandrogens

- Insecticide metabolites (p,p’-DDE, HPTE)
- Fungicides (Vinclozolin, Procymidone)
- Herbicide (Linuron)
- Some Imidazole and Triazole antifungals
- Phthalate esters (DBP; DEHP)
- Organophosphate pesticides (Fenitrothion)
Development of the Male Reproductive Tract

Bipotential Gonad
- Germ cell Precursor
- Supporting cell precursor
- Steroidogenic cell precursor

Testis
- gonocyte
- Sertoli Cell
- Leydig Cell

Male duct and organs
- Mullerian Duct Regression
- Wolffian ducts (epididymis, vas deferens, seminal vesicles)
- testicular descent
- External genitalia (penis, scrotum, prostate)

Signaling factors
- SRY
- MIS
- T (testosterone)
- DHT (dihydrotestosterone)
Development of the Male Reproductive Tract

**Rat**

- Testosterone
- Testicular descent
- Differentiation of external genitalia and prostate
- Wolffian duct differentiation
- Müllerian duct regression

**Human**

- Testosterone
- Testis descent
- Male external genital differentiation
- Wolffian duct differentiation
- Müllerian duct regression
- External genital growth

**Gestation (weeks)**

- 5
- 6
- 7
- 8
- 9
- 10
- 11
- 12
- 13
- 14
- 15
- 40

**Postnatal day**

- 0
- 20
- 40

**Gestation day**

- 12
- 14
- 16
- 18
- 20
Fetal Testis
Phthalates

- Widely used plasticizers that impart flexibility and strength to a variety of consumer goods and plastics
- Found in food packaging, clothing, paints, toys, and personal care products
- Anti-androgenic
- Does not bind to androgen receptor
Phthalates Disrupt Male Rat Reproductive Development

- Cryptorchidism
- Male reproductive tract malformations
  - Epididymal malformations
  - hypospadias
- Testicular dysgenesis
  - Regions of Leydig cell hyperplasia
  - Multinucleated gonocytes
  - Abnormal seminiferous cord formation
Decrease in Anogenital Distance Among Male Infants with Prenatal Phthalate Exposure


Environ Health Perspect
## Phthalate Profile Study

<table>
<thead>
<tr>
<th>Phthalate Ester</th>
<th>Reproductive effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethyl</td>
<td>-</td>
</tr>
<tr>
<td>Diethyl</td>
<td>-</td>
</tr>
<tr>
<td>Dioctyl-tere</td>
<td>-</td>
</tr>
<tr>
<td>Di-\textit{n}- butyl</td>
<td>+</td>
</tr>
<tr>
<td>benzobutyl</td>
<td>+</td>
</tr>
<tr>
<td>Di-\textit{n}- pentyl</td>
<td>+</td>
</tr>
<tr>
<td>Di-(2-ethylhexyl)</td>
<td>+</td>
</tr>
</tbody>
</table>
Phthalate esters

DEHP

DBP

DMP

DEP
Study Design

Gestation day: 12 16 19 21

Isolate and snap freeze testis for RNA isolation
Affymetrix microarray results

- Investigated approximately 30,000 genes
- 391 genes significantly altered (1.3%)
  - 225 unknown transcribed sequences
- DMP, DEP, DOTP
  - Not significantly different from control or each other.
- DBP, BBP, DPP, DEHP
  - All significantly different from control and DMP, DEP, DOTP
  - But not each other
391 unique genes

- Universal mean determined for each gene

- Red = increased relative to mean
- Green = reduced
Gene Ontology
167 genes

- Lipid, cholesterol, sterol homeostasis 31 (19 %) ↓
- Steroidogenesis 12 (7 %) ↓
- Lipid, cholesterol, sterol transport 10 (6 %) ↓
- Transcription Factors 9 (5 %)
- Signal Transduction 22 (13 %)
- Oxidative Stress 11 (7 %) ↓
- Cytoskeleton 13 (8 %)
- Unclassified 59 (35 %)
Steroidogenesis

Insulin signaling
Lipogenesis
Sterol transport
Transcription Factors

Ldlr
Vldlr
cd36

SR-B1
HDL

Cell Membrane

DBP

Cholesterol Synthesis
Cholesterol

DBP

Mitochondrial Membrane

StAR

P450scc

Pregnenolone
3β-HSD
Progesterone
17α-hydroxylase
17-OH progesterone
17, 20-lyase
Androstenedione
17β-HSD

CYP17

AR

Testosterone

PBR
DBI
P450 OR
ferredoxin
Substantial Reductions in Testosterone Occur Before Developmental Endpoints
Cholesterol transport

Scavenger Receptor

Relative Expression

mRNA

Protein

StAR

Relative Expression

Dose DBP (mg/kg)

Dose DBP (mg/kg/day)

Dose DBP (mg/kg/day)
DBP-induced cryptorchidism
Role of insl3 in Testicular Descent

- Intra-abdominal position
- Inguinal canal
- Normal position

Trans-abdominal descent (Insl3-mediated)
Location of androgen receptor antagonist-exposed testes
Scrotal descent (androgen-mediated)
Role of insl3 in Testicular Descent

- Intra-abdominal position
- Trans-abdominal descent (Insl3-mediated)
- Location of DBP-exposed testes
- Scrotal descent (androgen-mediated)

- Inguinal canal
- Normal position
- Scrotum
Phthalate-Induced Testicular Dysgenesis

Sertoli Cell
- Cholesterol
- Steroidogenesis
- Oxidative Stress
- Testosterone
- Reproductive tract development
- Testicular Descent

Gonocyte
- c-KIT
- FGF signaling

Reproductive tract development

LHR, CNP
- SRB1
- LDLR
- VLDLR
- CD36
- Cholesterologenesis
- Insl3
- α inhibin

Fetal Leydig Cell
Phthalate-Induced Testicular Dysgenesis

- ↓ SRB1
- ↓ LDLR
- ↓ VLDLR
- ↓ CD36

- Cholesterol
- ↓ Cholesterol
genesis
- ↓ Lipogenesis
- ↓ Insulin Signaling

- Steroidogenesis
- ↓ Oxidative Stress

- Fetal Leydig Cell

- Disrupted Cell Junctions
- ↓ GJA1
- ↑ Testin

- Sertoli Cell
- ↓ c-KIT
- ↓ FGF signaling
- ↓ Dax1

- Multinucleated Gonococyte

- Altered Sertoli Cell development
- ↓ α inhibin
- ↓ Testosterone
- ↓ Insl3

- Malformed Reproductive tract
- Cryptorchidism
Early gene changes in the fetal testis after DBP exposure
Time Course

- 500 mg/kg/day
- 0, 0.5, 1, 3, 6, 12, 18, & 24 h
- Between gd 18 and 19
- Testosterone RIA
- RT-PCR
- Microarrays at 1, 3, 6, 18 h
DBP causes a biphasic reduction in fetal testicular testosterone and gene expression

![Graph showing the reduction in testosterone concentration and relative expression over time.](image)
Time course heat map

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>DBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Time (h):
DBP induces immediate early gene expression
Promoter Analysis

Dnase footprinting
Chromatin Immunoprecipitation
EMSA
Western analysis
DBP inhibition of Testosterone biosynthesis pathway

![Graph showing testosterone concentration and relative gene expression](image-url)
Automated DNase I Footprinting

- DNA subjected to DNase digestion
- DNA regions bound by protein are protected from digestion
- DNA fragments are amplified with Ligation Mediated-PCR using promoter specific primers
Changes in StAR promoter architecture using in vivo DNase footprinting
Changes in StAR promoter architecture using in vivo DNase footprinting
EMSA

Promoter Location and Sequence (bp upstream)

Relative Fluorescence

Promoter Location and Sequence (bp upstream)
EMSA
Chromatin Immunoprecipitation quantifies changes in specific TF binding.
Proposed Mechanism
Proposed Mechanism
Proposed Mechanism
Effect of DBP on Mouse Fetal Testis
MBP Pharmacokinetics

Maternal Plasma - Single 500 mg/kg
- MBP-RAT-GD19
- MBP-MOUSE - GD 18

Fetal Plasma - Single 500 mg/kg
- MBP- Rat- GD 19
- MBP- Mouse GD 18
Gene Expression Following DBP Exposure

Lipid, Sterol, and Cholesterol homeostasis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mouse</th>
<th>Rat</th>
<th>Gene Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dhcr7</td>
<td>↑</td>
<td>↓</td>
<td>7-dehydrocholesterol reductase</td>
</tr>
<tr>
<td>Aacs</td>
<td>↑</td>
<td>↓</td>
<td>acetoacetyl-CoA synthetase</td>
</tr>
<tr>
<td>Clps</td>
<td>↓</td>
<td>NS</td>
<td>colipase, pancreatic - lipid catabolism</td>
</tr>
<tr>
<td>Cyp51</td>
<td>↑</td>
<td>↓</td>
<td>cytochrome P450, family 51</td>
</tr>
<tr>
<td>Hsd17b7</td>
<td>↑</td>
<td>↓</td>
<td>hydroxysteroid (17-beta) dehydrogenase 7</td>
</tr>
<tr>
<td>Idi1</td>
<td>↑</td>
<td>↓</td>
<td>isopentenyl-diphosphate delta isomerase</td>
</tr>
<tr>
<td>Lss</td>
<td>↑</td>
<td>↓</td>
<td>lanosterol synthase</td>
</tr>
<tr>
<td>Stard4</td>
<td>↑</td>
<td>↓</td>
<td>StAR-related lipid transfer domain containing 4</td>
</tr>
<tr>
<td>Scd1</td>
<td>↑</td>
<td>↓</td>
<td>stearoyl-Coenzyme A desaturase 1</td>
</tr>
<tr>
<td>Scd3</td>
<td>↓</td>
<td>---</td>
<td>stearoyl-coenzyme A desaturase 3</td>
</tr>
<tr>
<td>Sc5d</td>
<td>↑</td>
<td>↓</td>
<td>sterol-C5-desaturase</td>
</tr>
</tbody>
</table>
Multinucleated gonocytes in mouse fetal testis following DBP exposure

A  
Seminiferous cord diameter

B  
MNG / Cord

C  
Nuclei / MNG
DBP targets common and divergent pathways in the Mouse and Rat

- **Rat** → IEG → Leydig Cell
- **DBP**
- **Mouse** → IEG → Sertoli / germ cell
- Steroidogenesis
  - Reproductive tract Development
  - Testicular Descent
- Insl3
- Testicular Descent
- Cord Formation & MNG
- Testin
- FGF
- TSP1
- CTGF
- Integrin signalling
Testis transplant model

![Graph showing testosterone levels in Corn Oil and DBP groups.](image)
Application of Genomics to Reproductive Health Risks

Testicular Dysgenesis Syndrome

Additional Studies
- Dose-Response
- Time Course
- Profile
- Pharmacokinetics
- Species comparisons

High-throughput, Microarray analysis

Animal Studies Reproductive Tract

Systems Biology & Computational BB-DR Models

Dose-Response
Fetal Androgen Insufficiency

Decreased Testosterone → Decreased DHT → Block Androgen Receptor → Mutated Receptor

Decreased AR activity at target tissue

AR mediated reproductive tract malformations
Acknowledgments

- Kamin Johnson
- Adam Kuhl
- Elena Kleymenova - GSK
- Jack Liu - GSK
- Susan Ross
- Kim Lehmann
- Janan Hensley
- Delong Liu
- Kim Boekelheide - Brown
- Susan Borghoff - ILS
- Paul Foster - NIEHS
- Chris Thompson - Sanofi-Aventis
- Valerie Shultz - Sanofi-Aventis
- Stan Young - NISS
- Russ Wolfinger - SAS
- ACC-LRI
- NIH R01 ES011803-01A1
- NIH R21 ES011754-01
- EPA STAR R830766