

# Identification of Region-Specific Gene Expression Changes and Signalling Pathways Affected by Dibutyl Phthalate in Foetal Rat Testes.

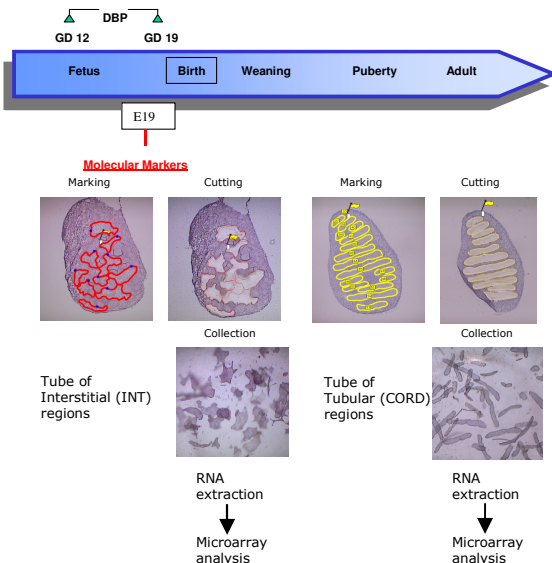
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## Introduction

*In utero* exposure of rats to high doses of certain phthalate esters causes testicular abnormalities such as cryptorchidism, hypospadias and reduced testicular testosterone levels. Phthalate-induced testicular disorders in rats arise due to abnormal development of Sertoli and Leydig cells. The mechanism(s) by which phthalate-induced testicular dysgenesis occurs is not yet fully understood. Transcription profiling of RNA isolated from laser capture microdissected interstitial/Leydig cell (INT) and tubular/Sertoli cell (TUB) regions from foetal testes of Wistar rats exposed *in utero* to di(*n-butyl*) phthalate (DBP) 500 mg/Kg was performed in order to identify gene expression changes and signalling pathways associated with DBP-induced testes mal-development. Determination of the regional-specificity of the gene expression alterations associated with DBP-induced testicular disorders may provide a better understanding of the molecular processes that underlie abnormal development of different cell-types following *in utero* exposure of foetal testes to DBP.

## Experimental outline



20-60 ng total RNA isolated from INT or TUB regions was labelled using the Low Input RNA Fluorescent Linear Amplification Kit (Agilent# 5184-3523) and hybridised on a Whole Rat Genome 60mer oligo microarray (Agilent #G4131A) containing approximately 40,000 genes. Separate hybridisations were performed with RNA from foetal testes of 3 different *in utero* DBP-exposed litters against a pool of RNA isolated from foetal testes of 3 different control (vehicle exposed) litters. We included 'dye' swap replicates giving a total of 6 microarrays (6 data points per gene) for each region.

Data from each group of 6 arrays was combined with an error weighted algorithm using Rosetta Luminator software. Signature genes from each region were selected as significantly 'regulated' if their combined P-value was less than 0.01. Rosetta Luminator software was used to compare the two signature gene lists. This analysis identified genes that were **uniquely or commonly** regulated by DBP in the two regions.

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## Transcription profiling results

Transcription profiling analysis identified genes that were uniquely or commonly regulated by DBP in the two regions. Effects on pathways regulating steroidogenesis (STAR), cholesterol synthesis (HMGCS, ID1), fatty acid oxidation (SCD) and testes morphogenesis (CRABP2, FAT, INHA) were focussed to the INT (Leydig cell) region, Table 1. By contrast genes involved in Mullerian duct regression (AMH), chromatin bending (HMG1, HMG2), phagocytosis (MARKS) and the response to hypoxia (HIF1A) were uniquely altered in the TUB (Sertoli cell) region. Genes that were identified as being commonly regulated by DBP in the two testes regions were associated with steroidogenesis (DBI, FABP5, SCARB1, Cyp 17A) and cell/tissue assembly (PHGDH, ARPCS, SERPING1).

## A

GenBank	Gene/Category	Fold Change	Description
<b>Steroidogenesis</b>			
NM_031558	STAR	-4.62	steroidogenic acute regulator
<b>Cholesterol synthesis</b>			
NM_017268	HMGCS1	-2.89	hydroxymethylglutaryl-CoenzymeA synthase
NM_031840	FDPS	-2.88	farnesyl diphosphate synthase (farnesyl pyrophosphate synthetase, dimethylallyltransferase, geranyltransferase)
NM_053539	ID1	-3.85	isopentenyl-diphosphate delta isomerase
<b>Fatty acid oxidation</b>			
AI175764	SCD	2.28	stearoyl-CoA desaturase (delta-9-desaturase)
<b>Cellular Development</b>			
NM_031819	FAT	-1.69	FAT tumor suppressor homolog 1 (Drosophila)
<b>Insulin receptor signalling</b>			
NM_022392	INSIG1	-9.93	insulin induced gene 1
AF087674	IRS2	1.81	insulin receptor substrate 2
<b>Antioxidant response</b>			
NM_031509	GSTA5	-2.33	Glutathione transferase A5
XM_215562	MGST2	-2.49	Microsomal glutathione transferase 2
<b>Cell growth and proliferation</b>			
AI045044	CDKN1C	-2.69	cyclin-dependent kinase inhibitor 1C (p57, Kip2)
<b>Gubernacular development</b>			
NM_053690	INSL3	-1.96	insulin-like 3 (Leydig cell)
<b>Testes Morphogenesis</b>			
NM_017244	CRABP2	-2.04	Cellular retinoic acid binding protein 2
NM_012590	INHA	-2.31	inhibin, alpha

## B

GenBank	Gene/Category	Fold Change	Description
<b>Tissue morphology</b>			
NM_012902	AMH	1.24	anti-Mullerian hormone
<b>Chromatin modelling</b>			
NM_012963	HMG1	-1.56	high-mobility group box 1
AW917230	HMG2	-1.29	high-mobility group nucleosomal binding domain 2
<b>Calcium signalling</b>			
NM_053867	TPT1	-1.57	tumor protein, translationally-controlled 1
<b>Phagocytosis</b>			
AW915660	MARKS	-1.92	myristoylated alanine-rich protein kinase C substrate
<b>Stress response</b>			
NM_024359	HIF1A	-2.48	hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
NM_031114	S100A10	-1.48	S100 calcium binding protein A10 (annexin II ligand, calpactin I, light calyopetide (p11))

## C

GenBank	Gene/Cat	INT Fold Change	TUB Fold Change	Description
<b>Steroidogenesis</b>				
NM_031853	DBI	-1.68	-1.87	dissepam binding inhibitor (acyl-Coenzyme A binding protein)
NM_145978	FABP5	-1.46	-1.38	fatty acid binding protein 5 (retinol-associated)
NM_031541	SCARB1	-3.7	-2.17	scavenger receptor class B, member 1
NM_012753	CYP17A1	-2.6	-2.22	cytochrome P450, family 17, subfamily A, polypeptide 1
NM_080886	SCAMOL	-4.91	-2.08	Sterol-C4-methyl oxidase-like
<b>Cell/tissue assembly</b>				
NM_031620	PHGDH	-1.34	-1.45	phosphoglycerate dehydrogenase
CF110878	ARPCS	-1.39	-1.38	actin related protein 2/3 complex, subunit 5, 16kDa
NM_199093	SERPING1	-2.22	-1.43	serine proteinase inhibitor, clade G (C1 inhibitor), member 1
<b>Cellular metabolism</b>				
NM_022592	TKT	-1.7	-1.42	transketolase (Wernicke-Korsakoff syndrome)
NM_017126	FDX1	-2.65	-2.08	Ferredoxin
<b>Tissue Morphology</b>				
NM_134334	CTSD	3.1	-1.44	cathepsin D (lysosomal aspartyl protease)

Table 1. Genes expression changes ( $p < 0.01$ ) at GD 19 that were (A) unique to the INT (Leydig cell) region; (B) unique to the TUB (Sertoli cell/gonocyte) region; and (C) common to both INT and TUB regions.

## Immunohistochemistry results

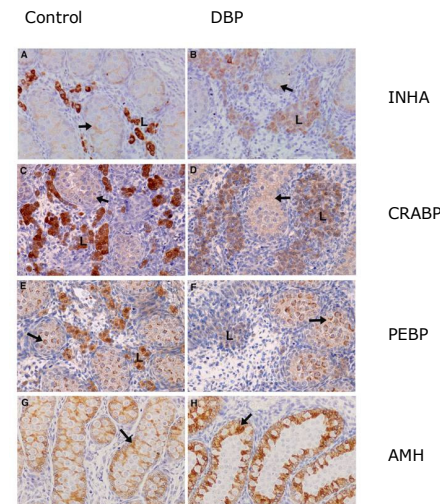


Figure 2: Immunostaining in foetal rat testes from control (left panels) and *in utero* DBP-exposed (right panels) rats - GD19. **INHA**: inhibin alpha, **CRABP**: cellular retinoic acid binding protein, **PEBP**: phosphoethanolamine binding protein; **AMH**: Anti Mullerian Hormone. Key: Arrows- Sertoli cells; L- Leydig cells.

## Summary and Conclusions

- Laser capture microarray analysis identified a number of genes that were uniquely or commonly altered in the INT or TUB regions.
- For 3 genes that are expressed in both Leydig- and Sertoli cells, namely **INHA**, **CRABP2** and **PEBP**, RNA expression was **only** altered (down-regulated) in the interstitial (Leydig cell) fraction. The cellular specificity of this effect was confirmed at the protein level by immunohistochemistry suggesting that microdissection procedure of the INT region was enriched with RNA from Leydig cells.
- Although there is data indicating that **CYP17** is only expressed in Leydig cells (Majdic et al *J Endocrinol*, 147: 285-293, 1995), our data suggests that expression of this gene at the RNA level is not confined to this cell type.
- Several genes in the INT region are regulated by nuclear receptors e.g **SF-1** (STAR, CYP17, INHA, HMGCS, INSL3); **PPAR $\alpha$**  (SCD); **PPAR $\gamma$**  (SCARB1, IRS2, FDPS); **RAR $\alpha$**  (CRABP).
- The data suggest that the inhibitory effects of DBP on SF-1-regulated genes are primarily on the INT (Leydig cell) region.

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