Targeted RNA-sequencing of testes from fetal rats exposed to dicyclohexyl phthalate informs potency and adverse outcome pathway development

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Background

- Dicyclohexyl phthalate (DCHP) is a known reproductive toxicant that has been correlated with testicular dysgenesis syndrome.
- Differential gene expression analysis can provide insight into the potential mechanisms of toxicity of DCHP.
- Templated Oligo-Sequencing (TempO-Seq) provides a streamlined and efficient means of analyzing gene expression compared to qRT-PCR and microarrays.

Purpose: Apply benchmark dose response modeling of TempO-Seq data to determine DCHP potency, compare results to custom qRT-PCR array data (Gray et al., 2021*), and inform DCHP mode of action.

- The TempO-Seq dataset will identify new and confirm previously identified dose-dependent, differentially expressed genes (DEGs) associated with DCHP from qRT-PCR dataset.
- Ingenuity pathway analysis will predict significant pathways that are known in DCHP toxicity.
- A potency estimate will be generated using benchmark dose analysis.

*Gray Jr., L.E., Lambright, C.S., Conley, J.M., Evans, N., Furr, J.R., Hannas, B.R., Wilson, V.S., Sampson, H., and Foster, P.M.D. (2021). Genomic and Hormonal Biomarkers of Phthalate-Induced Male Rat Reproductive Developmental Toxicity Part II: A Targeted RT-qPCR Array Approach That Defines a Unique Adverse Outcome Pathway. *Toxicological Sciences* 182(2), 195-214.

Experimental Design

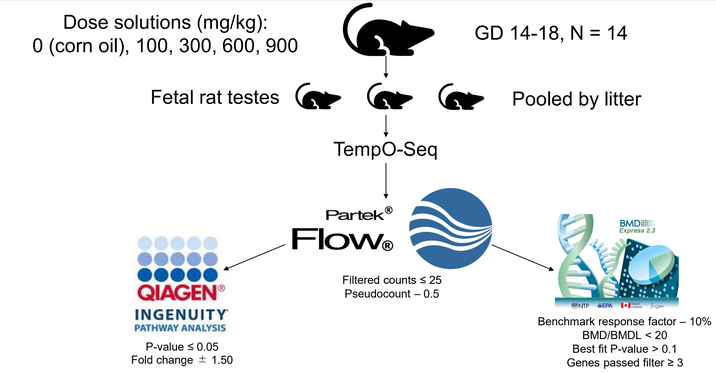


Fig. 1. Experimental overview demonstrating dam exposure, fetal testes collection, and analysis of the TempO-Seq dataset.

Despite variability in biological replicates, DCHP treatment-related differences apparent

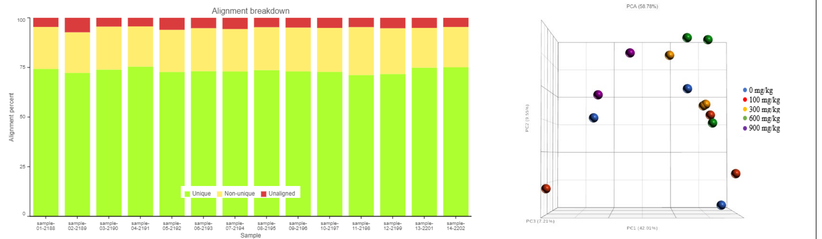


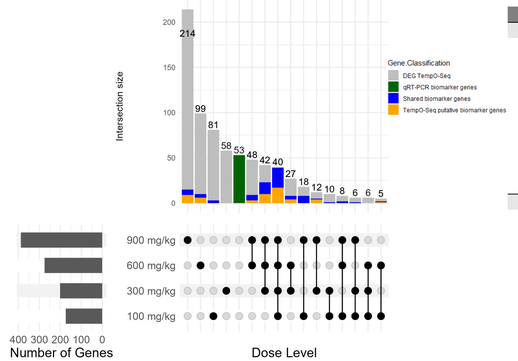
Fig. 2. The unique, non-unique, and unaligned sections using the STAR alignment with the PCA of each sample. Data was generated in Partek Flow.

TempO-Seq confirmed and identified new DEGs, including *Testin*

Putative biomarker genes unique to TempO-Seq				
ACAT2	CDKN1C	HMOX1	PPARGC1B*	TARDBP*
ACLY	CEBPA*	LIPG	PRLR	TESTIN
ACSS2	CEBPB*	LRP8*	PTPRZ1	TLR3
AGPAT2	CPA1	LRRC39	RBM47	TWIST1
ALDOC	DPT	LSS	SCD	XBP1
ASAH1*	EDN1	LYNX1	SIRT2*	ZNF296
B3GALT5	EGR2*	MET	SLC41A3	
BDNF	EPHX1	MMP13	SMAD3*	
BMP5	ESRRA*	MSMO1	SP1*	
CCN1	FABP3	NR5A2*	SQLE	
CD14	FDX1	PFKP	STC2	
CD68	HMG20A*	PLCH1	SVS5	

Shared biomarker genes between TempO-Seq and qRT-PCR					
ABCA1	CYP51	HSD3B2	NR0B1*	PPARA*	SOX9
ABCG1	DHCR7	IDI1	NR0B2*	PPARD*	SRA1*
ACAA2	DMRT1*	INHA	NR1H2*	PPARG*	SREBF1*
ACOX1	DVL3	INHBA	NR1H3*	PTCH1	SREBF2*
APOA1	ESR1*	INSIG1	NR1H4*	RARB*	SRY*
APOC1	ESR2*	INSL3	NR3C1*	RARG*	STAR
APOE	FDFT1	LHCGR	NR3C2*	RHOX5*	TGFB1
AR*	GATA4*	LHX1*	NR4A2*	RXRA*	TM7SF2
CBX2	HDLBP	LHX9*	NR5A1*	RXRG*	TRERF1*
CYP11A1	HMGCR	MAPK3	NSDHL	SCARB1	VDR*
CYP17A1	HMGCS1	MVD	PDGFRA	SFRP4	WT1*
CYP46A1	HSD3B1	MVK	POU5F1*	SOAT1	ZFPM2*

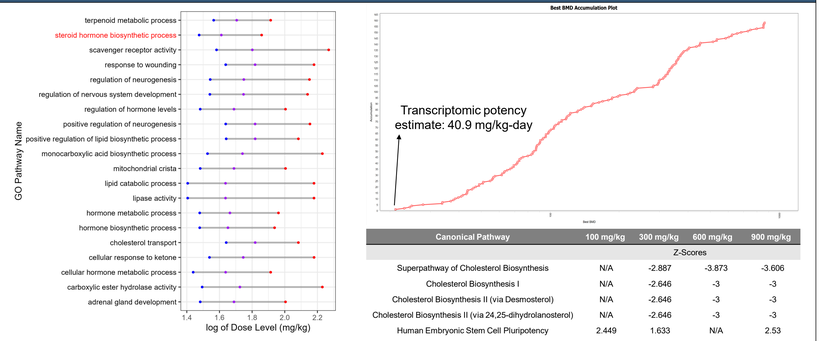
Biomarker genes unique to qRT-PCR arrays				
ACTB	DIXDC1	FGF9	PDGFA	TLE1
ACVR2B	DKK1	GUSB	PTGDS2	TLE2
ADH1	DKK3	HOXA2	RARA	TSPO
ALDH1A1	DMRT2	HSD17B3	RGD1563046	VLDLR
AMHR2	DVL1	INHBB	RGD1564999	WNT7A
AXIN1	DVL2	LDHA	RHOX10	
AXIN2	EBP	LDLR	RXRB	
B2M	ELA3B	LOC691504	SFRP1	
CYP11B1	EMX2	NR1D1	SFRP2	
CYP11B2	FABP1	NTF3	SFRP5	
CYP4A1	FDPS	NTRK3	SMO	
DHH	FGF8	PCAF	SOX8	



Gene Name	100 mg/kg	300 mg/kg	600 mg/kg	900 mg/kg
Top downregulated genes				
SVS5	-1.646	-4.665	-8.072	-22.852
CYP17A1	-1.755	-2.558	-4.452	-12.438
FDX1	-1.558	-2.63	-3.939	-5.851
STAR	-2.186	-3.55	-5.838	-5.642
SCARB1	-1.769	-2.912	-4.417	-4.198
LYNX1	-2.539	-2.916	-3.626	-4.118
CDKN1C	-1.7	-2.151	-2.768	-3.176
IDI1	-1.3	-2.004	-2.39	-3.119
STC2	-1.93	-2.401	-3.266	-3.045
HSD3B2	-1.493	-1.939	-1.986	-2.54
Top upregulated genes				
LRRC39	2.009	4.641	5.072	7.316
PLCH1	2.113	1.949	2.541	3.094
BMP5	1.856	1.896	2.084	2.514
LOC500567	1.676	1.938	1.704	1.987
CD55	1.349	1.405	1.445	1.663
DNASE2	1.317	1.229	1.385	1.322
ALKBH3	1.232	1.362	-1.250	1.314
GPAT2	1.205	1.128	1.190	1.213
RAPH1	1.154	1.164	1.24	1.212
HBP1	1.138	1.153	1.119	1.160

Fig. 3. Overlap of the genes between the TempO-Seq and qRT-PCR datasets and the top 10 upregulated and downregulated genes with fold changes. Data generated in IPA. *indicates these genes are upstream regulators.

Top biological pathways correlate with known DCHP mechanisms



Canonical Pathway	100 mg/kg	300 mg/kg	600 mg/kg	900 mg/kg
	Z-Scores			
Superpathway of Cholesterol Biosynthesis	N/A	-2.887	-3.873	-3.606
Cholesterol Biosynthesis I	N/A	-2.646	-3	-3
Cholesterol Biosynthesis II (via Desmosterol)	N/A	-2.646	-3	-3
Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)	N/A	-2.646	-3	-3
Human Embryonic Stem Cell Pluripotency	2.449	1.633	N/A	2.53

Fig. 4. The BMDU (red dot), BMD (purple dot), and BMDL (blue dot) values for the top 20 GO pathways with the lowest median BMD pathway highlighted in red, a table of the top canonical pathways, and the BMD accumulation chart. GO pathways and accumulation plot generated and analyzed in BMDExpress (v. 2.3) and canonical pathways analyzed in IPA.

Conclusions

- A potency estimate was developed that was close to, but slightly more sensitive, than a previously identified developmental and reproductive $BMDL_{10}$ estimate of 68 mg/kg-day.
- Certain genes changed expression levels in a dose-dependent manner, which is something that has been established for DCHP.
- Targeted RNA-Seq identified genes that weren't included in the qRT-PCR arrays, including *Testin.* These genes should potentially be monitored with the other known biomarker genes.
- Ingenuity pathway analysis identified potential mechanisms of action through analysis of pathways and upstream regulators that could have resulted in the downregulation of hormone regulation and synthesis.
- **Targeted RNA-Seq is an efficient means of collecting a large amount of transcriptomic data that can subsequently be used to inform chemical potency and mechanisms of toxicity.**

Acknowledgements

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