320# IMMUNOHISTOCHEMICAL AND CHIP MICROARRAY ANALYSIS OF PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR ALPHA (PPARα) IN FETAL RAT TESTES EXPOSED IN UTERO TO DIBUTYLPHTHALATE

Plummer, Simon M.[1]; Dan, Dhritiman[1]; Quinney, Joanne C.[1]; Millar, Michael[3]; Hallmark, Nina[2]; Phillips, Richard D.[2]; Elcombe, Clifford R.[1]
[1]CXR Biosciences, Dundee, United Kingdom, [2]ExxonMobil Petroleum & Chemical, Hermeslaan, Belgium & [3]MRC Human Reproductive Sciences Unit, Edinburgh, UK.

Abstract #1041

Introduction

Previous ChIP microarray analysis suggested that the inhibitory effects of dibutlyphthalate (DBP) on expression of the SF1-regulated gene Cyp11a was mediated by direct binding of PPARα to the Cyp11a promoter in proximity to SF1 binding sites at gestational day 15 (GD15) (Plummer *et al* (2010) J. Soc Toxicol 114:1488). As Cyp11a is important for testosterone synthesis in fetal Leydig cells we postulated that PPARα may be a target for the anti-androgenic effects of DBP. To test this hypothesis we performed;

(1) Immunohistochemical staining of GD15 fetal testes with antibodies against PPARα and the Leydig cell marker βHSD.

(2) Further ChIP microarray data analysis of SF1 and PPARα binding to SF1 regulated genes important for testosterone synthesis including StAR, Cyp17a and a 'negative control' gene FSHR, that is SF1 regulated but not affected by DBP at the RNA expression level (Plummer *et al* (2007) Toxicol Sci 97:520).

(3) ChIP analysis of transcription factor binding (CBP) to explore whether its availability could be a limiting factor.

(4) Comparison of these events at two developmental stages was made (GD 15 and 19).

Experimental Outline

Immunostaining was performed on Bouin's fixed GD15 fetal testes with antibodies against PPARα and βHSD to test for colocalisation of these two proteins.

ChIP microarray was performed on GD15 and 19 fetal testes from Wistar rats exposed from GD12 to GD19 *in utero* to DBP (500mg/Kg, p.o. to dams) as follows:
IP antibodies chosen, PPARα, SF1, CBP, RNA pol 2
Fix, lyse and fragment 'target' cells/tissue
- 8 fetal rat testes per sample (~80,000 cells)
- 4 biological replicates (litters)/treatment

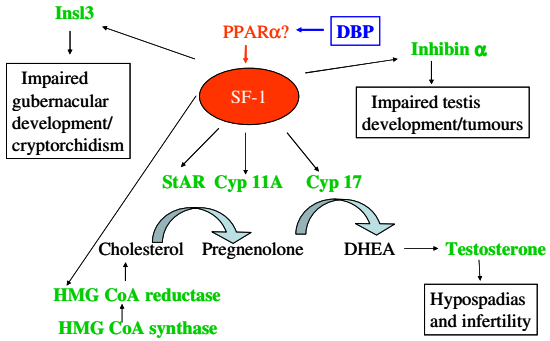
Agarose gel QC of fragmentation (200-500bp)
Aliquot sample to produce IP and input (reference) DNA samples
IP enrichment of 'target' genes
Amplify IP and input DNA samples using whole genome amplification (WGA) kits
QC for IP enrichment of 'target' genes using PCR/agarose gels or real time PCR with sybr green
Cy5/Cy3 Labeling and hybridisation of IP and input DNA on the ChIP array (created using e. Array).

Questions

What are the differences between the vehicle- (corn oil control) and DBP-treated fetal testes samples (GD15 and GD19) at the level of SF1, PPARα and/or CBP-binding to steroidogenic gene promoters?

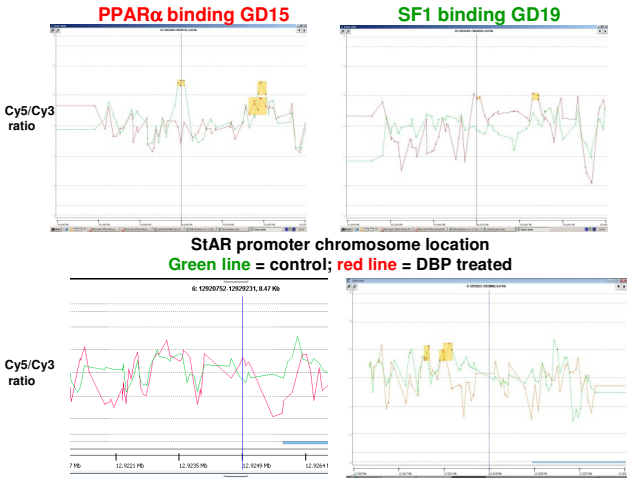
Do the changes in transcription factor/coactivator binding caused by DBP treatment correlate with changes in mRNA expression of these genes?

Fig. 1: Hypothesis: DBP-mediated inhibition of fetal testes steroidogenesis pathways (GD19)



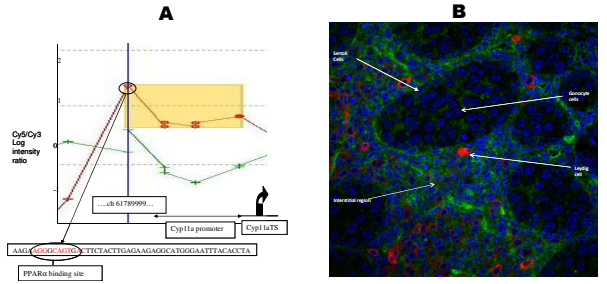
Hypothesis derived from our microarray-based transcription profiling analysis, for DBP induction of testicular mal development involving indirect repression of SF1 and SF1 target genes such as those involved in steroidogenesis, cholesterol synthesis, gubernacular development and aspects of male reproductive organ differentiation.

Fig. 2: In utero DBP treatment decreases SF1 binding and increases PPARα binding in the StAR promoter



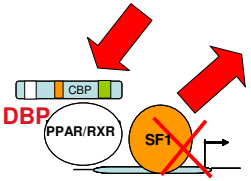
The binding of SF1 or PPARα to the StAR and FSHR promoters in control (green line) & in utero DBP-treated (red line) GD15 (left panels) and GD19 (right panels) fetal rat testes, respectively. Results are a mean of the data for each of the 4 replicates/treatment. The orange boxes show peaks as defined by intensity ratio differences between neighbouring probes as detected using the Whitehead peak detection algorithm. The presence of peaks\treatment differences are also evaluated by visual examination. The X axis represents the genomic position of probes (crosses). The Y axis is the Cy5/C3 fluorescence log intensity ratio (binding signal). DBP treatment inhibits binding of SF1 to the StAR promoter but not to the FSHR promoter. The inhibition correlates with increased PPARα binding to the StAR promoter. There were no PPARα peaks in the FSHR promoter. PPARα and SF1 impact on the StAR promoter are sequential (PPARα at GD15 & SF1 at GD19) and not concurrent (further data not shown). This is consistent with the idea that PPARα is an indirect not a direct transrepressor of SF1/StAR.

Fig. 3: Steroidogenic promoters contain PPARα 'consensus' binding sites and PPARα is expressed in Leydig cell in GD15 fetal testes



(A) PPARα bound probe in Cyp11a promoter contains a 'consensus' binding sequence matched at ~70% (red) (B) GD15 testis section showing interstitial region. DAPI stained cell nuclei (Blue). Alexafluor 555 stained Leydig cell marker 3βHSD (Red). FITC stained PPARα expressing cells (Green) GD15 testis showing co localisation of 3βHSD and PPARα in Leydig cells (blue arrows).

Fig. 4: Hypothesis for mechanism of DBP-induced PPARα-mediated SF1 transrepression in the steroidogenesis gene promoters



Cyp 11a promoter

PPARα binds in the steroidogenesis gene (Cyp11a, StAR, Cyp17a) promoter and disrupts the binding of SF1 such that transactivation is inhibited.

Conclusions

- **PPARα is expressed in the fetal rat testes at GD15.**
- **Increased PPARα binding at GD15 correlates with decreased expression of steroidogenesis genes.**
- **Inhibition of SF1 regulated steroidogenesis gene expression in fetal testes correlates with inhibition of SF1 binding to their promoters.**
- **SF1 is a 'target' for DBP-mediated repression of steroidogenesis genes.**
- **Inhibition of binding of SF1 to 'target' gene promoters at GD19 correlates with increased binding of PPARα at GD15 consistent with with the idea that PPARα is an indirect not a direct transrepressor of SF1/steroidogenesis genes.**

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