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Introduction

Understanding molecular mechanisms of *in vivo* toxicity as reflected by parallel changes in gene expression requires techniques that are capable of measuring alterations in cell-types that are relevant to the toxicity. To determine the utility of applying whole genome microarray-based transcription profiling analysis to laser microdissected tissue sub-regions for mechanistic studies of anti-androgen (AA)-induced rat fetal testes toxicity, we have compared data generated using 1 colour (1C) and 2 colour (2C) microarray methods. To facilitate an assessment of the utility of the 1C method for evaluating toxicological mechanisms compared to a 2C method we have evaluated the biological significance of the data generated by both methods using Ingenuity Pathways Analysis™.

Experimental Outline

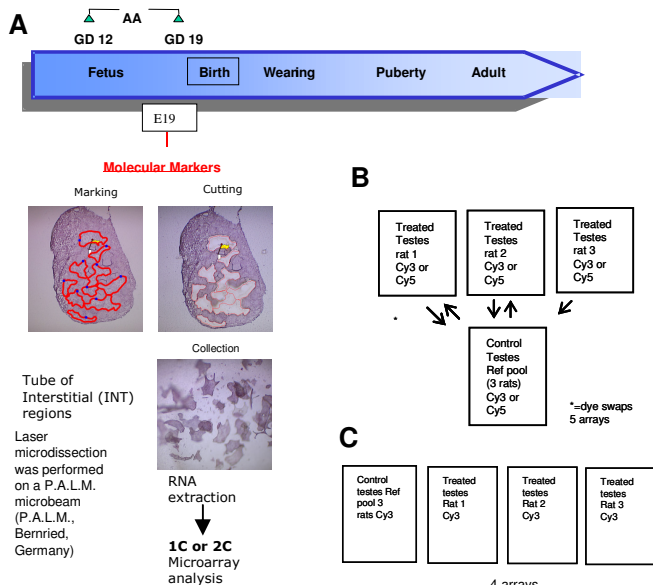


Figure 1A: Structure of experiment. Male rats were exposed *in-utero* to anti-androgen (AA) on gestational days (GD)12-19. Transcription profiling analysis was performed on RNA extracted from laser microdissected foetal testes INT regions at GD 19. B: 2C microarray experimental structure including hybridisations of Cy3 and Cy5 labelled RNA on one array and including dye swaps. C: 1C microarray experimental structure with single colour hybridisations (Cy3 only).

500pg RNA isolated from INT underwent 2 cycles of linear amplification with the EpicentreTargetAmp 2-Round aRNA Amplification Kit 2.0. aRNA was directly labelled with Cy3 or Cy5 using kreatech ULS reagents and hybridised on a Whole Rat Genome 60mer oligo microarray (Agilent #G4131A). 2C hybridisations were performed with RNA from fetal testes of 3 different *in utero* AA-exposed litters against a pool of RNA isolated from fetal testes of 3 different control (vehicle exposed) litters. We included 'dye' swap replicates giving a total of 5 microarrays (5 data points per gene) for each labelling method. 1C hybridisations included the same RNA samples used for the 2C hybridisation but in this case only Cy3 labelled RNA was used (4 microarrays). Rosetta Resolver™ software was used to combine expression ratio (log ratio) values from the individual 2C arrays to make an error weighted mean (n=5) and signature gene list of significantly $P < 0.001$ altered genes. Resolver was used to generate a signature gene list of significantly ($p < 0.001$) altered genes from the 1C arrays (4 microarrays) and to generate 'filtered' signature lists from the 1C and 2C microarray data. Ingenuity Pathways Analysis™ software was used to assess the biological significance of the gene expression changes in the 1C and 2C filtered signature lists.

Transcription profiling Results

Scatter plots highlighting significant ($p < 0.01$) changes in gene expression (signature gene lists) (Figure, 1 A) for one colour and two colour microarray data indicated that there was a greater number of differentially expressed genes in the one colour data (compared to two colour) at low intensity levels. As low intensity genes are known to be associated with greater error (Mutch et al 2002) we filtered the signature gene lists to exclude the low intensity genes (Figure 1 B).

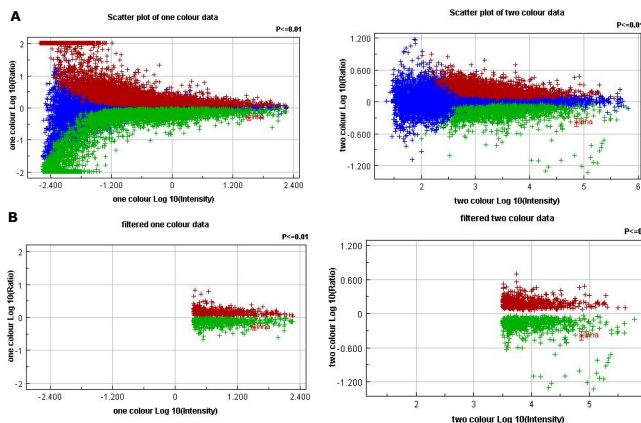


Figure 1. Scatter plots showing significantly ($p < 0.001$) altered genes (signature genes) caused by AA treatment. (A) : all signature genes (red=up-regulated, green=down-regulated); (B): filtered signature genes.

Comparison of 1C and 2C data using Resolver™ compare plot.

Correlation coefficient in filtered signature genes identified with the 1C and 2C protocols was ~ 0.7 , Figure 3

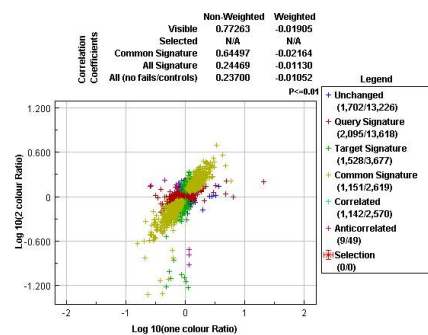


Figure 3. Resolver™ compare plots showing correlation between signature genes identified with 1C and 2C microarray methods. Correlated genes are highlighted in yellow.

Ingenuity Pathways Analysis Results

A comparison between the 1C and 2C signature genes showed that the same biochemical (canonical) pathways (ie lipid metabolism/sterol biosynthesis/Fatty acid biosynthesis) were identified as most significantly overrepresented in the two datasets, Figure 4. Networks associated with these biochemical pathways were also similarly highly represented in the two datasets (Figure 5).

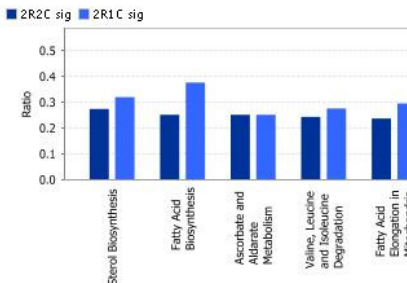


Figure 4. Histogram showing a comparison of biochemical (canonical) pathways assigned to genes that were overrepresented in the 1C and 2C datasets.

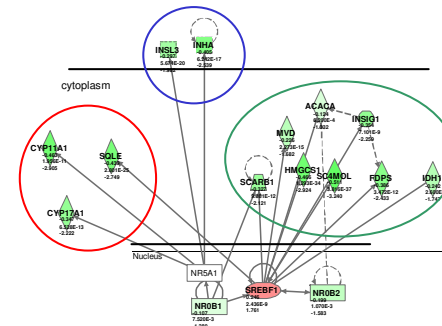


Figure 5. Merged IPA networks associated with testes descent and development (blue circle), sterol (green oval) and testosterone biosynthesis (red circle) for 1C and 2C analysis. Numbers under nodes are fold change and p values for 1C and 2C data, respectively.

Summary and Conclusions

- The 1C method identified $\sim 60\%$ more signature genes than the 2C method.
- $\sim 60\%$ of 'filtered' signature genes were common to the 1C and 2C signature lists
- Biological interpretation of the data generated by both methods reached similar conclusions.
- Due to differences in the numbers of signature genes identified by 1C and 2C methods, it would not be advisable to 'mix' the two methods in a single study.