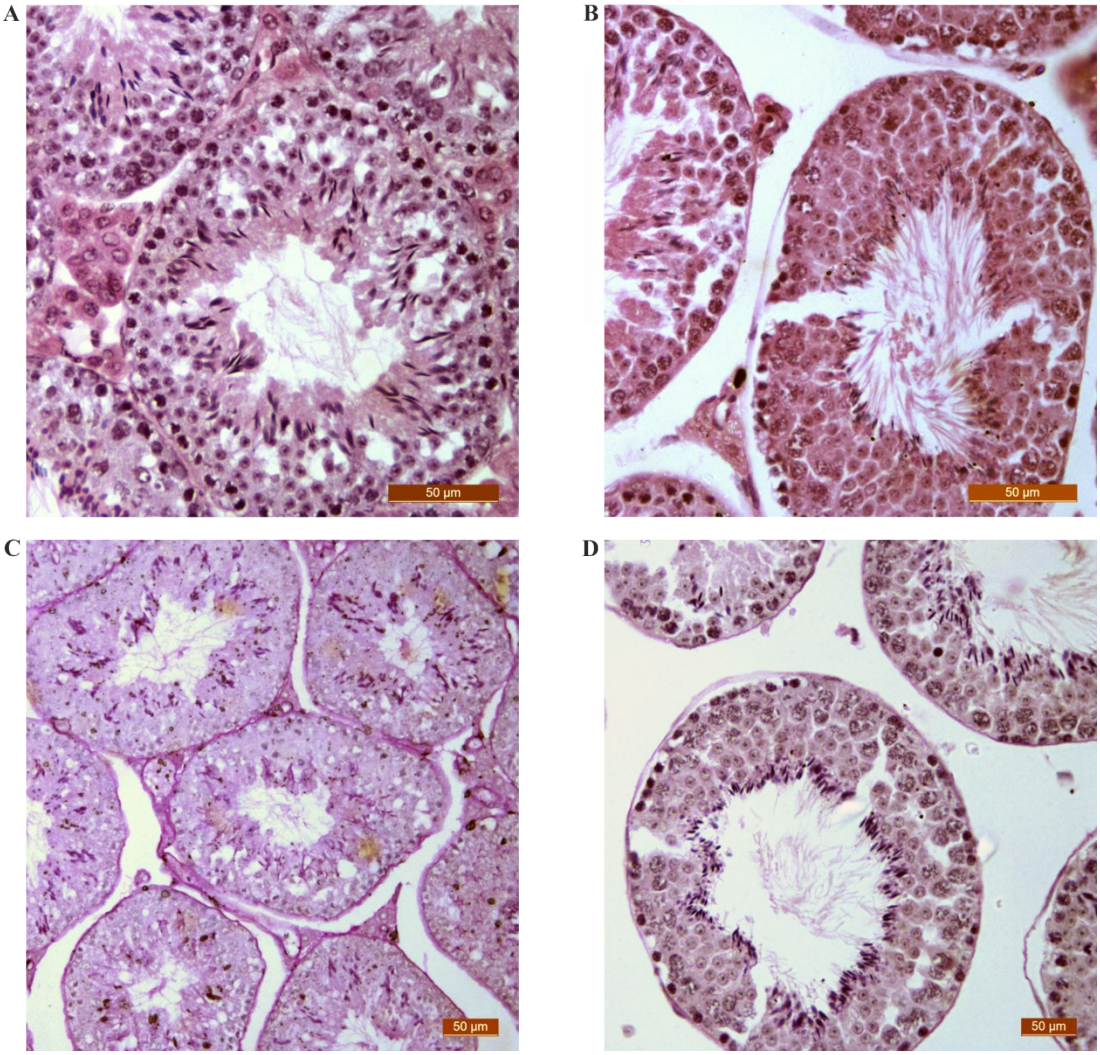
Electronic Supplementary Material (ESI) for Toxicology Research.

Perturbation of epigenetic processes by doxorubicin in the mouse testis

Oluwajoba O Akinjo, Timothy W Gant and Emma L Marczylo

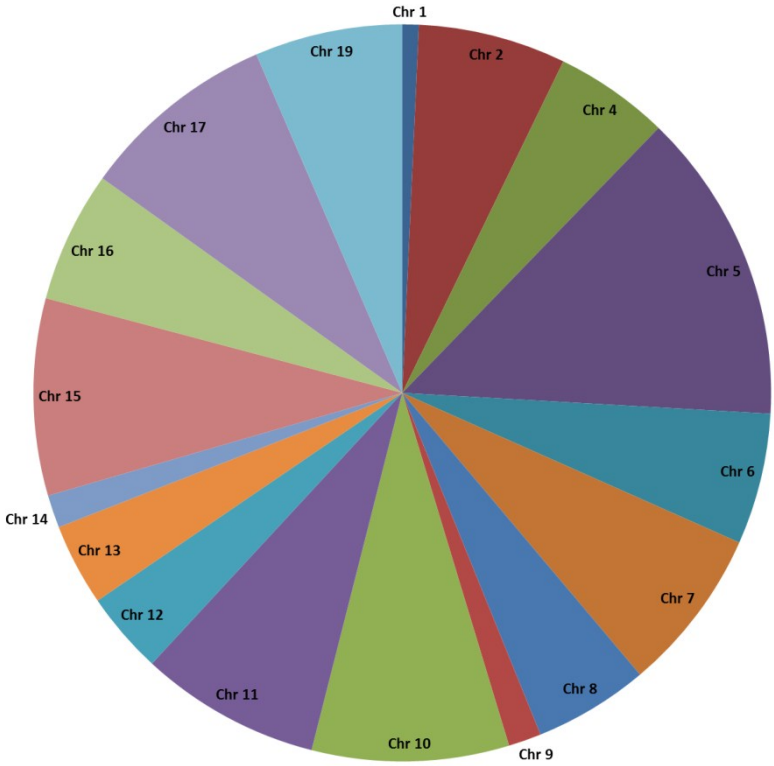
Contents: **Pages:**

Testicular sections were examined using two different fixatives (Bouin's and Zenker's), each with two different staining techniques (H&E and PAS) to ensure that all relevant cell types could be clearly identified and counted. H&E is a routine staining technique primarily used in histopathology to evaluate morphology, whilst PAS is a staining technique used to detect glycoproteins, which are present in acrosomes during the formation of spermatozoa from round spermatids. In H&E stained sections, the nuclear details of the different cells within the testis were more clearly defined in Bouin's-fixed testis compared to Zenker's-fixed testis (Supplementary Figure 1A-B). In PAS stained sections, the germ cells within the seminiferous tubules were more clearly stained in Zenker's-fixed testis compared to Bouin's-fixed testis (Supplementary Figure 1C-D). In particular, the contrast between elongated spermatids (dark purple) and other germ cells was more pronounced in Zenker's-fixed testis. Thus, Bouin's-fixed H&E stained sections were used for identifying the different testicular cell types, whilst Zenker's-fixed PAS stained sections were used for identifying and counting the germ cells at different stages of spermatogenesis.

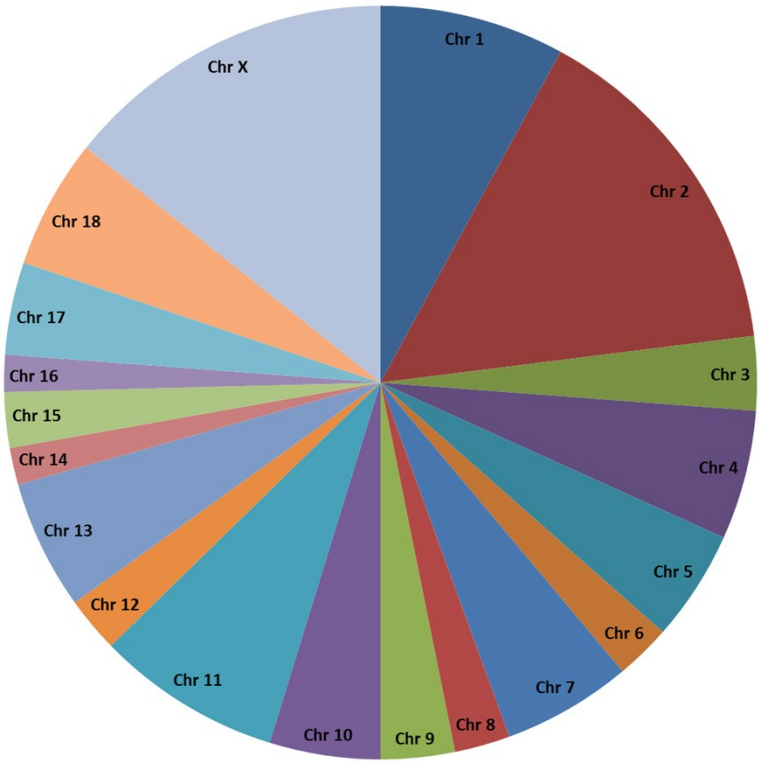


Supplementary Figure 1 Testicular histopathology A) H&E staining following fixation in Bouin's solution, B) H&E staining following fixation in Zenker's solution, C) PAS staining following fixation in Bouin's solution and D) PAS staining following fixation in Zenker's solution. Images are representative of control animals only.

A



B



Supplementary Figure 2 Chromosomal location of (A) the top 139 significantly DMRs in control animals ($p \leq 0.001$), and (B) the top 126 significantly DMRs over time following treatment with DOX ($p \leq 0.001$).

Supplementary Table 1 Primer sequences used for qRT-PCR

Transcript	Forward primer (3'-5')	Reverse primer (3'-5')
Bcl2	GTCGCTACCGTCGTGACTTC	GACCCCACCGAACTCAAAGAA
Casp3	GCACTGGAATGTCATCTCGCT	GGCCCATGAATGTCTCTCTGAG
Casp6	AAAAGTAGGGAAGTGTTCGATCC	CGAGTCAGGTTGTCTCTGTCTG
Catsper1	TTCAAGGAGGGACGAGTCTTAC	ATGGCTTGGGTCTAAGCTACC
Catsper3	CCTGGGATTCTGCCTATTTGG	AAGCCAGGTTCCCCCAGTT
Crem	ATGTCTTGAAAATCGTGTGGCT	TGGCAATAAAGGTCTTTGAGGG
Ctsb	TTGCGTTCGGTGAGGACATAG	TCCCGTGCATCAAAGGTTTCA
Ctsd	GCTTCCGGTCTTTGACAACCT	CACCAAGCATTAGTTCTCCTCC
Ctss	AGTGGGCATGAACGATATGGG	GTCAGGCAATGTCCGATTAGAG
Ctsw	CCTGGCTTCGTCCTCCTTC	GGCGATGACTGCATGGAGT
Cyp17a1	GCCCAAGTCAAAGACACCTAAT	GTACCCAGGCGAAGAGAATAGA
Dnmt1	TTCATGATGTGAAAAATGGCTACA	CCTTGCCTTCTGCACAGGAA
Dnmt3a	TTGTTGAGTCTAACCCCGTGATG	ATCATTCACAGTGGATGCCAAA
Dnmt3b	CCCGTTCGACTTGGTGATTG	CTTCCTGTGCCCTCATATAAACCT
Dnmt3l	GCTCTAAGACCCTTGAAACCCTTG	GTCGGTTCACTTTGACTTCGTA
Gapdh	CGTGTTCCTACCCCCAATGT	TGTCATCATACTTGGCAGGTTTC
Gstm3	AAGCACAACCTGTGTGGAGAGA	GCAGCAGACTATCATGAGCTGT
Hspa1a	TGGTGCAGTCCGACATGAAC	GCTGAGAGTCGTTGAAGTAGGC
Hspa2	TGAACCCCACAAACACCATCT	CGAACTTCCGTCCGATCAGC
Insl3	CATGCGCGCGCCGCTGCTAC	TCAGTGGGGACACAGACCC
Nme5	AAAACCCTAGCCCTTATCAAGC	AGGTGTAGTTTCCGTCTCTGAA
Stra8	GCCGGACCTCATGGAATTTGA	TCACTTCATGTGCAGAGATGATG
Tcf21	CCAACGACAAGTACGAGAACG	TCAGGTTGACTGGGTGAATGT
Tnp2	GGGAAAGTGAGCAAGAGAAG	ACTTGTATCTTCGCCCTGAGCT

Supplementary Table 2 qRT-PCR and array data of mRNAs and miRNAs of interest

Role	Transcript/miRNA	Time post DOX exposure			Time post DOX exposure		
		1wk	4wk	7wk	1wk	4wk	7wk
Testicular function	*Catsper1*	-4.2	-8.3	-5.0	-0.4	-1.7	-0.2
	Catsper3	-5.0	-6.7	-4.7	-0.4	-1.1	-0.4
	Crem	-1.7	-2.1	-1.5	-0.4	-0.5	-0.3
	Hspa2	-4.9	-4.8	-3.3	-1.3	-2.5	-0.6
	Nme5	-3.5	-5.7	-3.6	-0.4	-1.6	-0.5
	Stra8	-5.9	-5.5	-2.3	-0.4	-0.7	-0.3
	Tnp2	0.3	-5.8	-3.9	0.0	-3.8	-1.5
	Cyp17a1	1.0	0.8	0.9	0.4	0.8	0.6
	Insl3	2.1	2.4	2.1	0.7	2.6	1.3
Stress/cell death and survival	*Bcl2*	-0.2	1.2	1.5	0.0	0.6	0.7
	Casp3	0.6	1.5	1.4	0.3	0.5	0.7
	Casp6	1.0	1.4	0.6	0.3	0.8	0.4
	Ctsb	0.7	2.0	1.5	0.5	2.2	0.3
	Ctsd	0.7	1.4	2.0	0.3	0.3	0.4
	Ctss	1.3	2.2	2.7	0.4	1.9	0.4
	Ctsw	0.7	1.0	1.7	0.5	0.5	0.4
	Gstm3	1.3	1.9	3.5	0.7	1.7	0.8
	Tcf21	2.1	2.6	1.7	0.6	1.8	0.4
	Hspa1a	-4.8	-5.3	-2.7	-0.6	0.0	-0.5
DNA methylation and apoptosis	*Dnmt1*	-0.5	-1.3	0.3	-0.5	-0.3	0.0
	Dnmt3a	-3.4	-3.0	-1.2	-0.3	-0.8	-0.6
	Dnmt3b	-1.6	-1.6	-0.8	-0.3	-0.4	-0.6
	Dnmt3l	-5.2	-6.4	-3.5	-0.3	-0.4	-0.1
	miR-26a	0.1	1.8	1.3	0.1	1.1	1.0
	miR-29a	0.5	1.3	0.0	0.5	0.8	0.3
	miR-29b	0.5	1.6	0.6	0.6	1.0	0.5
	miR-29c	0.0	1.7	0.4	0.1	1.0	0.6
	miR-145	0.1	1.8	1.6	0.1	1.1	0.9
Method		**qRT-PCR data**			**Array data**		

mRNAs/miRNAs were selected due to either significant differential expression at more than one time point ($p \leq 0.001$) and from literature searches identifying their role in male infertility and tissue atrophy, and classified into groups based on their biological and physiological roles. Values represent mean Log_2[DOX/Control] ($n \geq 3$).

Supplementary Table 2 Correlation of DNA methylation and transcriptional profiles

Gene/Transcript	Time post DOX exposure			Time post DOX exposure		
	1wk	4wk	7wk	1wk	4wk	7wk
Hook1	**-2.1**	**0.9**	**1.1**	**0.2**	**-0.6**	**-0.3**
Spag16	**-2.5**	**0.1**	**0.9**	**0.0**	**-0.3**	**-0.3**
Stam	**-1.6**	**1.3**	**1.4**	**0.2**	**-0.3**	**-0.3**
Profile	DNA methylation			Transcription		

The 99 top most significantly DMRs associated with known genes were compared with the top 96 most significantly differentially expressed mRNAs ($p \leq 0.001$) to identify those with inverse correlations over time. Values for DNA methylation data represent mean Peak score ratio (Peak score DOX – Peak score control) ($n \geq 3$), and values for transcription data represent mean Log_2[DOX/Control] ($n \geq 3$). Decreased DNA methylation/mRNA expression is shown in green and increased DNA methylation/mRNA expression in red.