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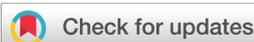**PAPER**
Emma L. Marczylo *et al.*
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spermatogonial, Leydig and Sertoli cell lines *in vitro*

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PAPER

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Perturbation of microRNA signalling by doxorubicin in spermatogonial, Leydig and Sertoli cell lines *in vitro*†

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We have previously shown that in addition to its widely recognised cardiotoxicity, the chemotherapeutic doxorubicin (DOX) is able to induce transcriptional, microRNA (miRNA) and DNA methylation changes in the mouse testis. These changes perturb pathways involved in stress/cell death and survival and testicular function and lead to germ cell loss and reproductive organ damage. Here, we further investigated the differential miRNA expression induced by DOX in mouse spermatogonial (GC1), Leydig (TM3) and Sertoli (TM4) cell lines *in vitro*. We began by performing cell cycle analysis of the three mouse testicular cell lines to evaluate their sensitivity to DOX and thus select suitable doses for miRNA profiling. In keeping with our *in vivo* data, the spermatogonial cell line was the most sensitive, and the Sertoli cell line the most resistant to DOX-induced cell cycle arrest. We then further demonstrated that each cell line has a distinct miRNA profile, which is perturbed upon treatment with DOX. Pathway analysis identified changes in the miRNA-mediated regulation of specialised signalling at germ–Sertoli and Sertoli–Sertoli cell junctions following treatment with DOX. Amongst the most significant disease categories associated with DOX-induced miRNA expression were organismal injury and abnormalities, and reproductive system disease. This suggests that miRNAs play significant roles in both normal testicular function and DOX-induced testicular toxicity. Comparison of our *in vitro* and *in vivo* data highlights that *in vitro* cell models can provide valuable mechanistic information, which may also help facilitate the development of biomarkers of testicular toxicity and high-throughput *in vitro* screening methods to identify potential testicular toxicants.

Introduction

There are now millions of cancer survivors worldwide. Current statistics estimate that 2.5 million people in the UK are living with, or have had treatment for, cancer.[1] This figure is estimated to increase by over three per cent each year, so that by 2030, there could be 4 million cancer survivors in the UK.[1] Around 1 in 4 people in the UK suffer from poor health or disability after cancer treatment.[2] The continued medical support of such survivors, particularly survivors of childhood cancers, is therefore an important Public Health issue. Childhood cancer survivors have greater life-years at risk of developing secondary cancers and chronic conditions in later life as a result of their previous treatments, and are also more likely to want to go on to have their own children. Leukaemias and lymphomas are the most common cancers diagnosed before the age of 25 in the UK.[3] Treatment of these cancers normally involves DOX or another anthracycline.[4] Thus, of the 35 000 survivors of childhood cancers in the UK,[3] around half will have been treated with an anthracycline.[4]

Anthracyclines are associated with lifelong cardiotoxicity,[5] and the extensive array of mechanistic studies (both from ourselves[6,7] and others[8–10] are helping to develop protective intervention strategies).[5,9,10] In contrast, much less is known about the long term effects of DOX, or other anthracyclines, on reproduction and development. It is not only the adverse outcome(s) on the treated cancer patient themselves that is of concern, but also the potential detrimental effect(s) on their future progeny. Over the last decade, many hundreds of studies have linked a wide range of environmental factors with adverse health outcomes in later life that can be transmitted across multiple generations, giving rise to so-called multigenerational toxicity.[11] Such effects are likely to involve epigenetic processes, including histone modification, DNA methylation and/or non-coding RNAs (ncRNAs) such as miRNAs. Indeed, a number of recent independent studies have implicated spermatozoal miRNAs in the transmission of stress-induced behavioural and metabolic abnormalities through subsequent

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generations.[12–15] There is also evidence to suggest that unique epigenetic information can be transmitted in the male germline *via* histone modifications and DNA methylation,[16–18] both of which help to activate/regulate early embryo development and can be perturbed by environmental factors.[11] Furthermore, increased multigenerational genomic instability following paternal anti-cancer drug exposure has been reported in mice.[19] Thus, there is concern that chemotherapeutics may induce epigenetically-mediated multigenerational reproductive and developmental toxicity *via* the male germline.[20]

The production of mature male germ cells (spermatogenesis) takes place within the seminiferous tubules of the testis and involves proliferation and differentiation of diploid spermatogonia into haploid spermatozoa.[21–24] Spermatogenesis is a complex developmental process supported by the secretion of hormones and other cellular signals from Leydig and Sertoli cells.[21–24] Compound-induced damage to any of these three main testicular cell types (germ, Leydig or Sertoli cells) could reduce the production of healthy spermatozoa, impair fertility and/or adversely affect the resulting embryo. While studies in rodents have associated DOX exposure with reduced testicular blood flow and volume, testicular atrophy, increased cellular apoptosis, impaired spermatogenesis, increased sperm abnormalities, sperm counts and fertility, and adverse developmental phenotypes in subsequent zygotes,[25–35] the molecular mechanisms underlying these DOX-induced effects are not well understood. Therefore, we recently investigated the molecular transcriptomic and epigenetic changes in the mouse testis following DOX treatment *in vivo*, linking specific miRNA, DNA methylation and mRNA changes to actual phenotypic endpoints.[36] Here we have further investigated the role of miRNAs within the three main testicular cell types in DOX-induced testicular toxicity by profiling miRNA expression in mouse spermatogonial, Leydig and Sertoli cell lines following treatment with DOX *in vitro*. We decided to focus on miRNAs since; (1) differential miRNA expression was better at separating the *in vivo* DOX treated samples compared to altered transcript expression,[36] and (2) spermatozoal miRNAs have been implicated in the transmission of phenotypes across generations.[12–15] The resulting *in vitro* data highlighted the roles of specific miRNAs, pathways and cell types during DOX-induced testicular toxicity.

Materials & methods

Materials

All materials, including DOX (as doxorubicin hydrochloride) were purchased from Sigma Aldrich, UK unless otherwise stated.

Cell lines

Three mouse testicular cell lines were used. The TM3 (Leydig) and TM4 (Sertoli) cell lines, originally derived from BALB/c mice testis, were gifts from the European Collection of Cell Cultures (ECACC) operated by Public Health England (PHE)

(Porton Down, UK), and the GC1 (spermatogonia) cell line, also derived from BALB/c mouse testis, was obtained from the American Type Culture Collection (ATCC) *via* LGC Standards (Teddington, UK).

Cell culture

The TM3 and TM4 cell lines were cultured in a 1 : 1 mixture of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F12 containing 17.5 mM glucose and 0.5 mM sodium pyruvate, supplemented with 5 mM glutamax, 5% horse serum and 2.5% foetal bovine serum (Life Technologies, Paisley, UK). The GC-1 cell line was cultured in DMEM containing 25 mM glucose and 1 mM sodium pyruvate, supplemented with 10% foetal bovine serum (Life Technologies, Paisley, UK). Cells were incubated in 5% CO_2 at 37 °C. Media was replaced every 2 days and cells were passaged when they reach a confluency of 80%.

Treatment with DOX

Approximately 1×10^4 cells were seeded in a 6-well plate and allowed to attach overnight. The media was then replaced with fresh media containing 1 nM–5 μM DOX or vehicle-only (PBS). After a further 24 or 48 hours of incubation, cells were collected for fluorescence-activated cell sorting (FACS) analysis or RNA extraction.

FACS analysis

Following treatment, cells were washed twice with PBS, trypsined, collected in media and centrifuged at 200*g* for 4 minutes at RT. Supernatant was discarded and the cell pellet was resuspended in 10 ml of ice cold fixing solution (1 ml PBS and 9 ml 70% ice-cold ethanol) for 24 hours at 4 °C. Fixed cells were centrifuged at 200*g* for 4 minutes at RT, the supernatant was discarded and the cell pellet was resuspended in staining solution (40 μg ml^{-1} propidium iodide (PI) and 1 mg ml^{-1} RNase A in PBS) at 37 °C for 30 minutes. Cell samples were then filtered through 70 μm mesh sieves to ensure a single cell suspension and analysed using a FACSCalibur Flow Cytometer (BD Biosciences, US). Forward scatter and side scatter detectors were gated to exclude as much debris as possible and histograms of fluorescence intensity (PI) *versus* number of cells were analysed using ModFit LT software (Verity Software House, USA). These histograms provide a representation of DNA content and thus the percentage of cells within the G1, S and G2 phases of the cell cycle. Representative examples of cell cycle profiles for each cell line following all treatments are shown in ESI Fig. 1–6.† The proportion of cells in G2 arrest was calculated as the percentage of cells in the G2/M phase (Dip G2, ESI Fig. 1–6†). Final percentages of cells in G2 arrest were normalised by subtracting the percentages of vehicle-only treated cells in G2 arrest, and plotted (mean ± SEM, $n \geq 3$) against Dox concentration (nM) to generate EC_{50} values and select low and high doses for RNA extraction and miRNA profiling studies.

RNA extraction

Following treatment, cells were washed twice with PBS, trypsined, collected in media and centrifuged at $200g$ for 4 minutes at RT. Supernatant was discarded and RNA was extracted from the cell pellet using the miRNeasy Mini kit (Qiagen, Germany) according to the manufacturer's instructions. RNA was stored at −80 °C until microarray analysis. RNA integrity was determined using a 2100 Bioanalyser and RNA 6000 LabChip® kit (Agilent Technologies, USA) according to the manufacturer's instructions. Only RNA samples with an RNA integrity number (RIN) ≥ 8.0 were used for downstream analysis.

miRNA profiling

Global changes in miRNA expression were profiled as described in our previous *in vivo* study[36] using miRNA microarrays obtained from the Medical Research Council Toxicology Unit (Leicester, UK) with the Label IT® miRNA labelling kit (Mirus, USA), except that all samples were run against a reference sample (instead of randomly pairing control *versus* treated samples and incorporating a dye-swap). The reference sample was made by mixing an equal amount of RNA extracted from all treated and untreated samples. The reference sample was labelled with Cy3 and hybridised against control or treated samples labelled with Cy5. Thus, expression values represent $\log_2$[cell line control/reference], $\log_2$[DOX/control] or fold change[DOX/control].

Bioinformatic analysis

Raw data was extracted, normalised and statistically significant differentially expressed miRNAs were identified as described in our previous *in vivo* study[36] using GenePix pro 6.0 (Molecular Devices, CA), the Bioconductor limma library package of R version 2.15.2 (http://www.r-project.org/) and Qlucore Omics Explorer (QOE) version 3.3 (Qlucore, Sweden), respectively. Heatmaps were subjected to hierarchical clustering using the algorithms within QOE.[37] Lists containing the top 100 most significantly differentially expressed miRNAs within/between the three testicular cell lines in the presence/absence of DOX were imported into Ingenuity® Pathway Analysis (IPA®) Software (Ingenuity® Systems http://www.ingenuity.com/products/ipa) to explore the more general functional roles of these miRNAs. In addition, lists containing the top 100 most significantly expressed miRNAs in the three testicular cell lines treated with DOX were merged and uploaded into miRwalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/) to identify experimentally validated mRNA targets. The resulting list of validated mRNA targets was then downloaded from miRwalk and imported into IPA® software to explore more specific networks and pathways involved in DOX-induced testicular toxicity.

Raw data files for all three cell lines have been submitted to GEO: GC1 (GEO***), TM3 (GEO***) and TM4 (GEO***).

Results

DOX induced cell cycle arrest

Cell cycle analysis of the three mouse testicular cell lines, GC1 (spermatogonia), TM3 (Leydig cells) and TM4 (Sertoli cells), in the presence or absence of 1 nM–5 μM DOX for 24 or 48 hours was performed to evaluate their sensitivity to DOX and thus select suitable doses for miRNA profiling. Representative examples of cell cycle profiles for each cell line following all treatments are shown in ESI Fig. 1–6.† All three cell lines demonstrated increased G2/M phase arrest following treatment with increasing doses of DOX for 24 (Fig. 1A) or 48 hours (ESI Fig. 7†). GC1 spermatogonia were the most sensitive to DOX-induced G2/M arrest, followed by TM3 Leydig cells and finally TM4 Sertoli cells (Table 1). No discernible differences were observed in the cell cycle profiles between DOX treatment for 24 and 48 hours,
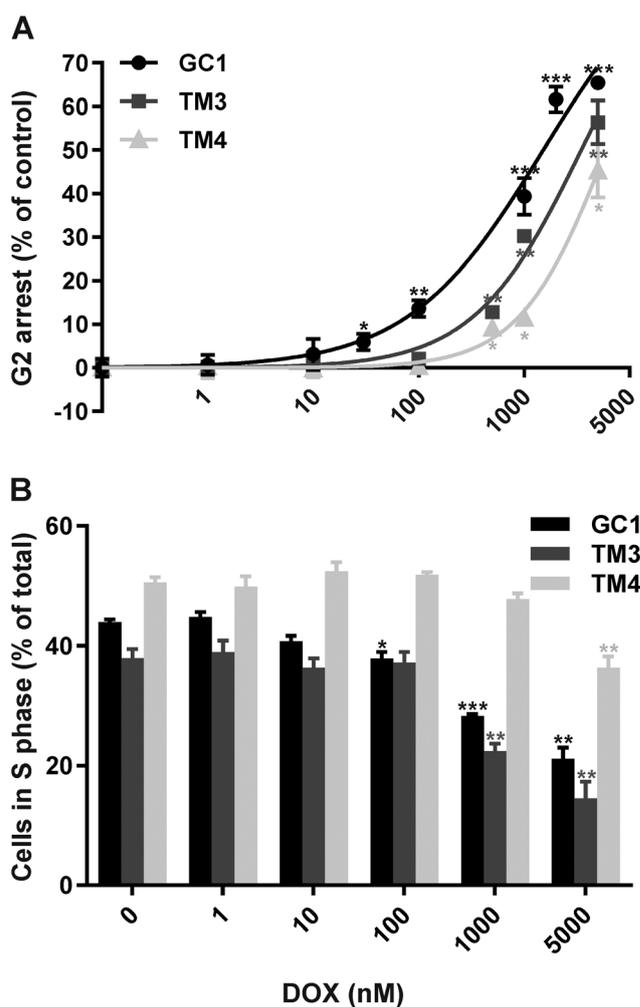

Fig. 1 Dose−response of (A) G2/M arrest or (B) actively dividing (S phase) cell loss following treatment with DOX for 24 hours. Cellular DNA content was analysed by FACS, and normalised by subtracting control values (percentage of cells in G2/M phase in the absense of DOX) from treated values (A) or calculated as a percentage of the total number of cells (B). Values represent mean ± SD ($n = 3$). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ (T-test compared to vehicle only).

Table 1 EC_{50} values for DOX-induced G2 arrest in the three testicular cell lines

Exposure time (hours)	GC1 Spermatogonia (μM)	TM3 Leydig (μM)	TM4 Sertoli (μM)
24	1.5	3.5	6.0
48	1.2	3.2	5.0

A dose–response curve was fitted to the data and used to calculate EC_{50} values in GraphPad prism.

except for a higher proportion of post-G2/M cells from 1–5 μM at 48 hours (indicative of cell aggregation) (ESI Fig. 1–6†), and an increased response at 48 compared to 24 hours (Table 1). Thus, 24 hours exposures were selected for downstream analyses.

The rate of DNA replication within each cell line, (as a measure of the percentage of S phase cells), was also compared (Fig. 1B). In the absence of DOX, TM4 Sertoli cells had the highest percentage of cells in S phase (50.5 ± 1.1%), followed by GC1 spermatogonia (44.0 ± 0.6%) and finally TM3 Leydig cells (38.0 ± 1.9%). All three cell lines demonstrated a reduction in the percentage of S phase cells following treatment with increasing doses of DOX for 24 hours. GC1 spermatogonia were the most sensitive to DOX-induced loss of S phase cells, followed by TM3 Leydig cells and finally TM4 Sertoli cells. The dose of DOX required to induce a statistically significant ($p < 0.05$) decrease in the percentage of S phase was 100 nM in GC1 spermatogonia, 1 μM in TM3 Leydig cells and 5 μM in TM4 Sertoli cells.

In summary, treatment with DOX induced significant G2/M arrest and loss of actively dividing (S phase) cells, with GC1 spermatogonia showing greatest sensitivity and TM4 Sertoli cells greatest resistance to DOX.

All three testicular cell lines demonstrated cell-specific miRNA profiles

We first compared the miRNA profiles of the three testicular cell lines in the absence of DOX. There was statistically significant ($p < 0.01$) differential expression of over 160 miRNAs, with the TM3 Leydig cells demonstrating the lowest level of change. The top 100 most statistically significant changes between the three cell lines, corresponding to 96 miRNAs, are shown in Fig. 2. Functional analysis of these 96 miRNAs using IPA® software revealed roles in various molecular and cellular functions, particularly cellular development, cell cycle, cell-to-cell signalling and interaction, and cellular assembly and organisation (covering approximately 55%, 20%, 15% and 5% of the total identified functional categories, respectively).

In summary, the three testicular cell lines demonstrated cell-specific miRNA profiles, with the most differentially expressed miRNAs predominantly functioning in cellular development and interaction.

DOX induced dose- and cell-dependent miRNA expression changes in all three testicular cell lines

Following cell cycle analysis of DOX-induced G2/M arrest, two doses (a low and high) were selected for each testicular cell
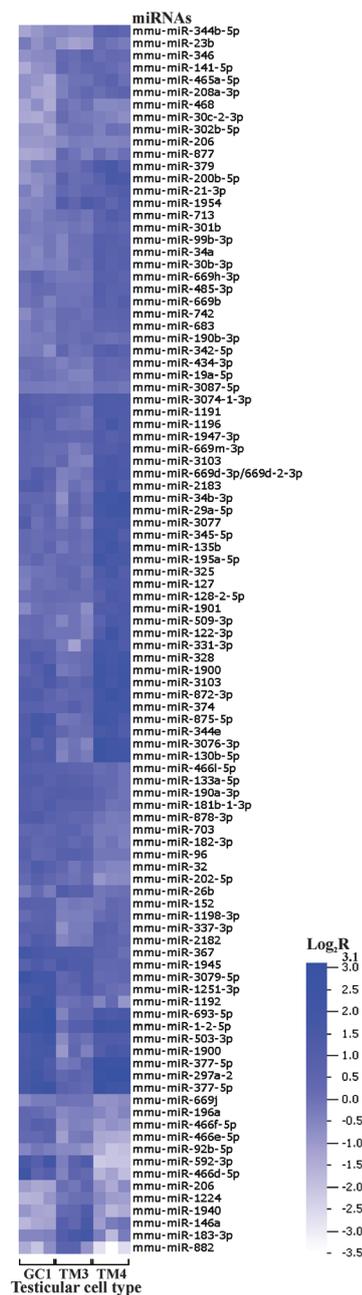

Fig. 2 Heatmap of the top 100 most significantly altered miRNAs ($p \leq 0.01$) between the three testicular cell lines GC1 spermatogonia, and TM3 Leydig and TM4 Sertoli cells. Expression levels represent $\log_2$[cell line/reference] ($\log_2 R$).

line. The low dose (100 nM for GC1 spermatogonia, and 500 nM for TM3 Leydig and TM4 Sertoli cells) marked the onset of G2/M arrest, while the high dose (1 μM for GC1 spermatogonia, and 5 μM for TM3 Leydig and TM4 Sertoli cells) induced significant G2/M arrest with minimal cell death.

There was statistically significant ($p < 0.01$) differential expression of between 60–120 miRNAs in the three cell lines following treatment with a low or high dose of DOX for 24 hours. TM4 Sertoli cells demonstrated the greatest number

(117) of changes, closely followed by GC1 spermatogonia (101), and finally TM3 Leydig cells (64). The top 100 most statistically significant changes with increasing dose, corresponding to 96 (GC1, $p < 0.01$), 95 (TM3, $p < 0.05$) or 93 (TM4, $p < 0.01$) miRNAs, are shown in Fig. 3. Approximately one third of these DOX-induced miRNAs were present in two or more cell lines, with only two (miR-767 and miR-3072) present in all three cell lines (ESI Fig. 8†). miR-767 was downregulated with increasing dose in all three cell lines, while miR-3072 was upregulated in GC1 spermatogonia but downregulated in TM3 Leydig and

TM4 Sertoli cells with increasing dose. Indeed, only 12 of the DOX-induced miRNAs present in two or more cell lines were altered with increasing dose in the same direction (ESI Table 1†).

The top 96, 95 and 93 most statistically significant ($p < 0.05$) DOX-induced miRNAs in each cell line were then independently analysed using IPA® software. This revealed roles in various molecular and cellular functions, including cellular development, cell cycle, cell-to-cell signalling and cell proliferation/death, with subtle differences in DOX-induced miRNA-
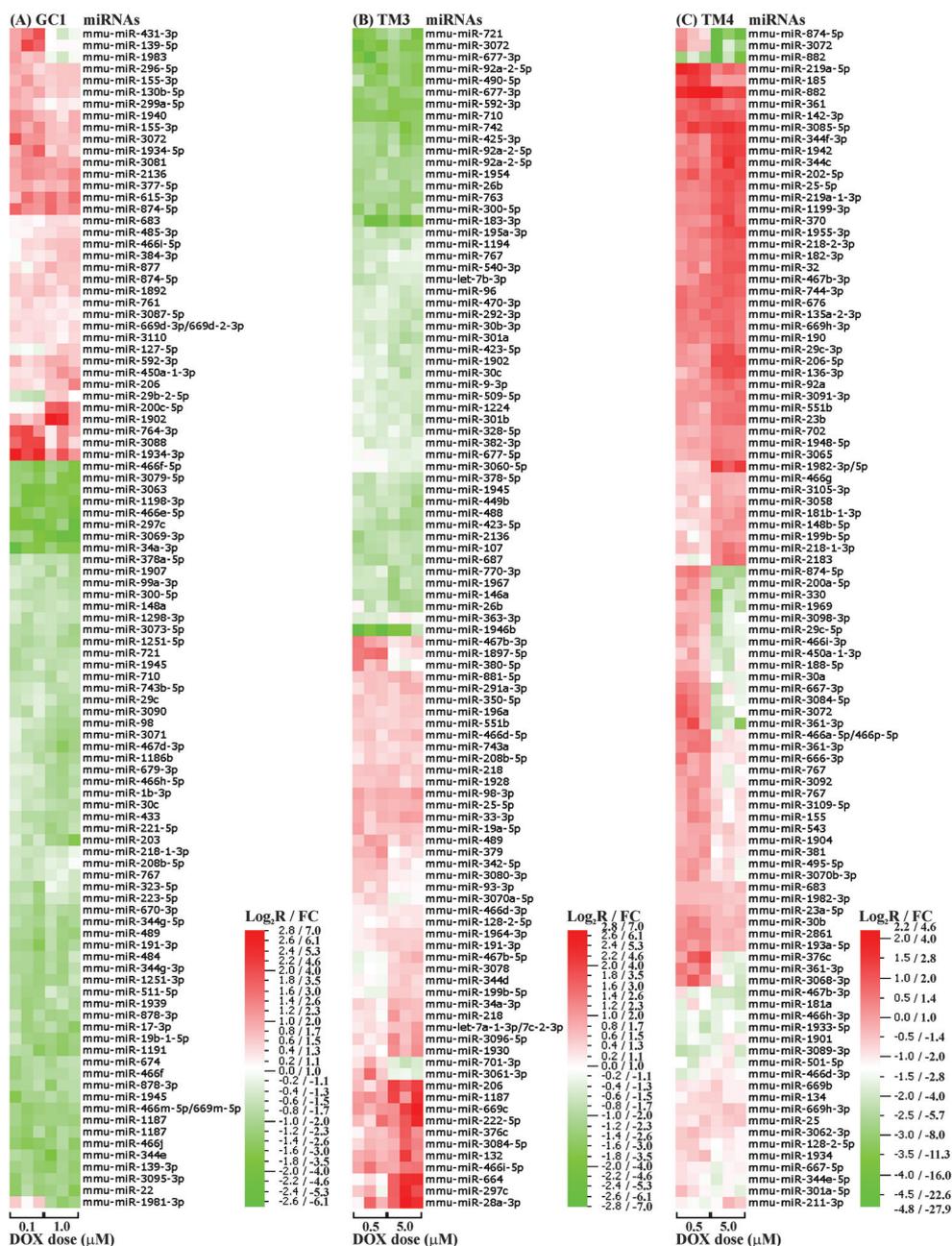

Fig. 3 Heatmap of the top 100 most significantly altered miRNAs ($p \leq 0.05$) within the three testicular cell lines (A) GC1 spermatogonia, and (B) TM3 Leydig and (C) TM4 Sertoli cells following treatment with DOX. Expression levels represent log₂[DOX/control] (Log$_2$ R) or fold change[DOX/control] (FC), with downregulations shown in green and upregulations in red.

associated functional categories between the different cell lines (ESI Fig. 9†). Although the cell cycle accounted for between 10–20% of the total functional categories in GC1 spermatogonia and TM3 Sertoli cells, it was not identified as a functional category in TM4 Sertoli cells. In contrast, the cell movement and morphology categories together represented approximately 30% of the total functional categories in TM3 Leydig cells, but were absent in GC1 spermatogonia or TM4 Sertoli cells. There was also a decrease in the cell communication functional category in GC1 spermatogonia and TM4 Sertoli cells compared to TM3 Leydig cells. Similarly, while cell proliferation and cell death/ survival accounted for approximately 50% of the total functional categories in GC1 spermatogonia and TM4 Sertoli cells, they were absent in TM3 Leydig cells. In all three cell lines, organismal injury and abnormalities and reproductive system disease were among the top three/four disease categories associated with DOX-induced miRNA changes.

Finally, the validated mRNA targets of the top 96, 95 and 93 most statistically significant ($p < 0.05$) DOX-induced miRNAs in each cell line were merged and analysed using IPA® software. Two signalling pathways were identified with specific functions in cell–cell interaction/communication during testicular development: germ cell–Sertoli cell junction signalling (Fig. 4A) and Sertoli cell–Sertoli cell junction signalling (Fig. 4B).

In summary, treatment with DOX induced dose- and cell-dependent miRNA changes *in vitro* that were associated with developmental disorders and reproductive system disease, and focussed around perturbation of germ cell–Sertoli cell and Sertoli cell–Sertoli cell communication.

DOX-induced miRNA changes *in vitro* can be correlated with those *in vivo*

Comparison of the DOX-induced miRNA expression profiles from the three *in vitro* testicular cell lines with that from the *in vivo* testis reported in our previous study[36] identified 15 miRNAs present in both the *in vivo* and *in vitro* systems (Table 2). miR-767 was present in the *in vivo* testis and all three *in vitro* testicular cell lines, being downregulated following treatment with DOX. Of the remaining 14 DOX-induced miRNAs, 3 were common to the *in vivo* testis and either the *in vitro* GC1 spermatogonia (miR-1892, 200c-5p and 486-3p) or the TM4 Sertoli cells (miR-330, 361-3p and 744-3p), while 8 were common to the *in vivo* testis and the *in vitro* TM3 Leydig cells (miR-1224, 146a, 1954, 323-5p, 382-3p, 687, 742, and 9-3p). The majority of these DOX-induced miRNAs (12/15) were altered in a direction that mechanistically correlated.

In summary, DOX induced a number of miRNA changes *in vitro* that could be mechanistically correlated with DOX-induced miRNA changes *in vivo*.

Discussion

In addition to its widely recognised cardiotoxicity, DOX can cause male reproductive toxicity.[25–35] We have previously
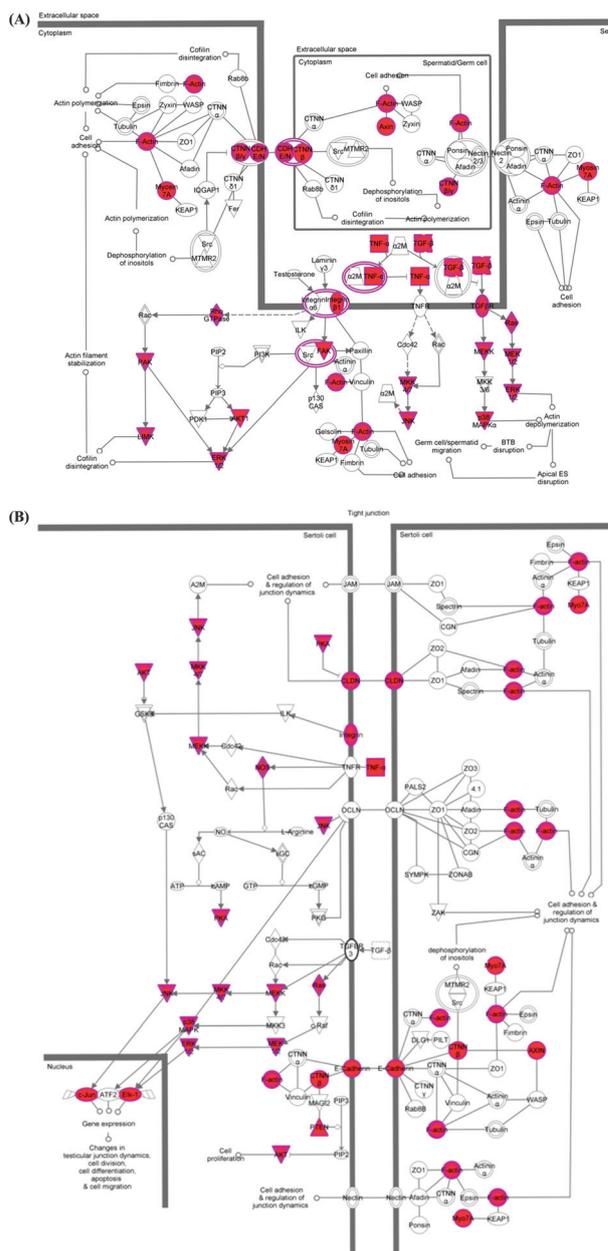

Fig. 4 The two signalling pathways identified with specific functions in cell−cell interaction/communication during testicular development (A) germ cell−Sertoli cell junction signalling and (B) Sertoli cell−Sertoli cell junction signalling. Validated mRNA targets of the most statistically significant ($p < 0.05$) DOX-induced miRNAs are highlighted in pink.

shown that DOX is able to induce transcriptional, miRNA and DNA methylation changes in the mouse testis, which perturb pathways involved in stress/cell death and survival and testicular function and lead to germ cell loss and reproductive organ damage.[36] This is of concern because (1) DOX is a widely used chemotherapeutic often used in children and young people who are more likely to want to go on to have their own children,[3,4] (2) epigenetic changes (such as altered DNA methylation and miRNA expression) have been linked to adverse

Table 2 DOX-induced miRNAs present in both the *in vivo* testis and one or more of the *in vitro* testicular cell lines

miRNA	*In vivo*			GC1 spermatogonia		TM3 Leydig cells		TM4 Sertoli cells	
	1 week	4 weeks	7 weeks	0.1 µM	1 µM	0.5 µM	5 µM	0.5 µM	5 µM
mmu-miR-1224	0.5/1.4	0.5/1.5	0.2/1.1			−0.4/−1.3	−0.6/−1.5		
mmu-miR-146a	0.0/1.0	−0.2/−1.1	−0.2/−1.1			−0.8/−1.7	−1.1/−2.2		
mmu-miR-1892	0.1/1.1	0.8/1.8	0.4/1.3	0.7/1.6	0.6/1.5				
mmu-miR-1954	0.0/1.0	−0.4/−1.3	−0.2/−1.1			−1.0/−2.0	−1.1/−2.1		
mmu-miR-200c-5p	−0.1/−1.1	−0.3/−1.3	0.0/1.0	0.2/1.1	1.6/3.1				
mmu-miR-328-5p	−0.1/1.1	0.3/1.2	0.3/1.3			−0.5/−1.4	−0.4/−1.4		
mmu-miR-330	0.0/1.0	−0.3/−1.3	0.1/1.1					−0.1/−1.1	−2.3/−5.0
mmu-miR-361-3p	−0.3/−1.2	0.3/1.2	0.4/1.3					0.5/1.4	−1.6/−3.0
mmu-miR-382-3p	0.0/1.0	−0.4/−1.3	−0.1/−1.1			−0.4/−1.3	−0.4/−1.3		
mmu-miR-485-3p	−0.1/−1.1	−0.3/−1.3	0.1/1.1	0.3/1.1	0.6/1.5				
mmu-miR-687	0.3/1.2	0.0/1.0	−0.1/1.0			−1.0/−2.0	−0.8/−1.8		
mmu-miR-742	0.2/1.1	−0.2/−1.2	0.0/1.0			−1.1/−2.2	−1.5/−2.8		
mmu-miR-744-3p	0.0/1.0	0.5/1.4	0.0/1.0					0.5/1.4	0.8/1.8
mmu-miR-767	0.5/1.4	0.1/1.1	−0.2/−1.2	−0.5/−1.4	−0.3/−1.2	−0.6/−1.5	−0.2/−1.2	0.0/1.0	−1.1/−2.2
mmu-miR-9-3p	0.3/1.2	−0.1/−1.1	0.1/1.1			−0.5/−1.4	−0.5/−1.5		

miRNAs were selected due to their significant differential expression ($p < 0.05$) in both the *in vivo* testis and one or more of the *in vitro* testicular cell lines following treatment with DOX. *In vivo* values represent mean log_2[DOX/control] or fold change[DOX/control] ($n = 6$). *In vitro* values represent mean log_2[DOX/control] or fold change[DOX/control] ($n = 3$). Black boxes represent absence of significant differential expression.

health outcomes resulting from environmental factors,[11] and (3) spermatozoal miRNAs have been implicated in the transmission of phenotypes across generations.[12–15] Thus, having identified DOX-induced miRNA perturbation in the mouse testis *in vivo*,[36] we wanted to further explore the differential miRNA expression induced by DOX in mouse spermatogonia, Leydig and Sertoli cell lines *in vitro* to assess the role of miRNAs within the different cell types in DOX-induced testicular toxicity.

Numerous *in vitro* studies have demonstrated that DOX induces cell cycle arrest at the G2/M checkpoint in a wide range of cell types.[38–42] Thus, we performed our own dose–response analysis of DOX-induced cell cycle arrest in three testicular cell lines representative of the cell types we investigated *in vivo*: germ (GC1 spermatogonia), Leydig (TM3) and Sertoli (TM4) cells. We chose these three cell lines as they were all isolated from the same mouse strain (Balb/c), and spermatogonia as the representative germ cell as these were more sensitive to DOX treatment than the more mature germ cells *in vivo*.[36]

DOX induced increasing dose-dependent G2/M arrest in all three testicular cell lines (Fig. 1A and Table 1). In keeping with our *in vivo* results, GC1 spermatogonia were the most sensitive to DOX-induced G2/M arrest, followed by the TM3 Leydig and finally the TM4 Sertoli cells. We have discussed potential reasons for the differing sensitivities of testicular cells in our previous publication, including the protective effects of physiological barriers, the expression of different cell membrane transporters and the varied replication rates of different testicular cell types.[36] The blood-testes or blood-epididymis barriers, both of which offer protection to the more mature germ cells (spermatocytes/spermatids) *in vivo*, are not present within our single cell type *in vitro* cultures. Moreover, *in vivo* spermatogonia (the most DOX sensitive, but least mature germ cells), Leydig and Sertoli cells are all located outside these physiological barriers. Thus barrier protection does not contribute to the differing sensitivities of these three specific cell types to DOX, *in vivo* or *in vitro*. In contrast, differences in the types and/or levels of membrane efflux pumps expressed by different testicular cell types are likely to contribute to the final intracellular DOX levels and thus the differing sensitivities of spermatogonia, Leydig and Sertoli cells both *in vivo* and *in vitro*. Determining the exact transporter profile of these three cell types and their intracellular DOX levels would provide further insight. Likewise, differences in cell replication rates are also likely to contribute to the differing sensitivities of spermatogonia, Leydig or Sertoli cells to DOX both *in vivo* and *in vitro*. Since DOX intercalates DNA and inhibits DNA synthesis, it would be expected to have a greater effect on more proliferative cells. Prior to any *in vitro* treatment, TM4 Sertoli cells had the greatest proportion of cells in S (replicating) phase, followed by GC1 spermatogonia and TM3 Leydig cells (Fig. 1B). Yet, GC1 spermatogonia were the most sensitive to DOX-induced loss of S phase cells, followed by TM3 Leydig cells and finally TM4 Sertoli cells (Fig. 1B). This suggests that although there was a greater proportion of TM4 Sertoli cells in replication phase at any one time *in vitro*, their rate of DNA synthesis remained slower than the GC1 spermatogonia cells. Sertoli (and Leydig) cells are terminally differentiated and do not normally undergo mitotic division in the sexually mature testis. Thus, even when forced into continuous proliferation as single cell monolayers, the spermatogonia, Leydig and Sertoli cells maintained their characteristic sensitivity profiles, highlighting that simple *in vitro* systems can model specific *in vivo* endpoints.

In vitro data can also offer mechanistic insights. As already described, we were particularly interested in the differential miRNA expression within the three testicular cell lines in the absence and presence of DOX. In the absence of DOX, all three

testicular cell lines demonstrated cell-specific miRNA profiles (Fig. 2), with the most differentially expressed miRNAs predominantly functioning in cellular development and interaction. This likely mimics the specific and differing roles of spermatogonia, Leydig and Sertoli cells *in vivo*. During spermatogenesis, Leydig and Sertoli cells support the proliferation and differentiation of spermatogonia into spermatozoa.[43,44] Leydig cells provide endocrine secretions (androgens, including testosterone),[43] while Sertoli cells provide endocrine, nutrient and direct physical support.[44] That the lowest level of change was observed in the TM3 Leydig cells suggests miRNA-mediated regulation plays a greater role in germ and Sertoli cells, particularly with respect to cellular interaction.

Cell-specific miRNA changes were also observed in the presence of DOX, some of which showed some dose-dependence (Fig. 3). Although organismal injury and abnormalities and reproductive system disease were identified as the major disease categories associated with DOX-induced toxicity in all three cell lines, there were subtle differences in the DOX-induced miRNA-associated functional categories between the different cell lines (ESI Fig. 9†). The absence of the cell cycle as a miRNA-associated functional category within TM4 Sertoli cells treated with DOX may help to explain the increased resistance of TM4 Sertoli cells to DOX-induced G2/M arrest compared to GC1 spermatogonia and TM3 Leydig cells. The presence of cell movement and cell morphology as DOX-induced miRNA-associated functional categories within TM3 Leydig cells may be linked to the apparent change in location, packing and number of the Leydig cells observed *in vivo* as the seminiferous tubules collapse following treatment with DOX.[36] Similarly, the presence of cell proliferation and cell death/survival as DOX-induced miRNA-associated functional categories within GC1 spermatogonia and TM4 Sertoli cells may be linked to the germ cell loss observed *in vivo* following treatment with DOX.[36] The DOX-induced reduction of the cell communication miRNA-associated functional category in GC1 spermatonia and TM4 Sertoli cells compared to TM3 Leydig cells, together with the increased number of miRNAs common to GC1 spermatogonia and TM4 Sertoli cells (ESI Fig. 8†), highlights that communication within and between germ cells and Sertoli cells may be a key miRNA-mediated process disrupted by DOX. That almost double the number of DOX-induced miRNA changes were observed in the GC1 spermatogonia and TM4 Sertoli cells compared to the TM3 Leydig cells further supports miRNA-mediated communication between germ and Sertoli cells as a key target of DOX-induced testicular toxicity.

miRNAs are known to target multiple mRNAs and therefore form large interactive networks that regulate complex pathways of gene expression. To perform a more comprehensive analysis of the types of networks and pathways perturbed by DOX *in vitro* the validated mRNA targets of the top 100 most significant DOX-induced miRNAs in each cell line were merged and subjected to pathway analysis. This identified two pathways important in cellular communication between Sertoli cells and Sertoli and germ cells (Fig. 4), providing further evidence that DOX perturbs Sertoli and germ cell communications.

Comparison of the *in vitro* and *in vivo*[36] miRNA profiles also provides some mechanistic insights, particularly the contribution of different cell types to DOX-induced miRNA changes and the potential roles of these miRNA changes within different cell types during DOX-induced testicular toxicity (Table 2). Of the 15 DOX-induced miRNAs present within the *in vivo* model and at least one of the *in vitro* testicular cell lines, 1 (miR-767) was common to, and downregulated across, all systems. This suggests all three cell types contribute to the loss of miR-767 within the testis following treatment with DOX *in vivo*. Interestingly, human miR-767 has been shown to target and repress expression of *ten-eleven translocation* (*Tet*) mRNAs *in vitro*.[45] The TET family of enzymes are involved in the active demethylation of DNA. They catalyse the oxidation of 5-methylcytosine to 5-hydroxymethylcytosine (and then 5-formylcytosine and finally 5-carboxylcytosine), all of which can either be passively depleted through DNA replication or actively reverted to cytosine through thymine DNA glycosylase-mediated base excision repair.[46] Reduction of miR-767 could therefore lead to increased TET levels and thus DNA demethylation. This may contribute to the DNA demethylation observed in the testis following DOX treatment *in vivo*.[36] The remaining 14 miRNAs were present within the *in vivo* and just one of the *in vitro* cell line systems. While 4 miRNAs were common to the *in vivo* testis and either GC1 spermatogonia or TM4 Sertoli cells, 9 were common to the *in vivo* testis and TM3 Leydig cells. The majority (12) of these changes can be mechanistically correlated, indicating the cell type from which the DOX-induced miRNA change(s) may originate *in vivo*. For example, while miR-200c-5p and 485-3p were upregulated with increasing dose in GC1 spermatogonia *in vitro*, they were initially downregulated over 1 and 4 weeks post-treatment before returning to normal or even increasing at 7 weeks post-treatment *in vivo*. DOX-induced spermatogonial cell loss in the testis followed the same pattern; spermatogonia (and spermatocytes and round spermatids), absent at both 1 and 4 weeks post-treatment, began to reappear at 7 weeks post treatment. This suggests that GC1 spermatogonia are the main source of these DOX-induced miRNA changes *in vivo*. Overexpression of miR-485-3p has been shown to increase DNA Topoisomerase (TopII) expression in human laeukaemia cells *in vitro*.[47] Since DOX is a TopII inhibitor, it is plausible that miR-485-3p is upregulated in an attempt to produce more TOPII in these mitotically active cells. Similarly, miR-330 and miR-744-3p were downregulated and upregulated, respectively, with increasing dose in TM4 Sertoli cells *in vitro* and at 4 weeks-post treatment *in vivo*. By 7 weeks post-treatment, both of these miRNAs returned to more normal levels, possibly signifying recovery of the Sertoli cells and/or rebuilding of Sertoli and germ cell communications as the germ cells begin to repopulate the testis. miR-744-3p has been shown to inhibit components of the DNA damage and repair response, leading to delayed DNA repair in human prostate cancer cells *in vitro*.[48] Since Sertoli cells do not appear to undergo cell death in the presence of DOX either *in vitro* or *in vivo*, it is possible that DOX-induces the upregulation of miR-744-3p, which inhibits

the DNA damage and repair response and signals a more senescent-like phenotype. Finally, Leydig cells appear to be the main source of DOX-induced changes in the expression of miR-9-3p, 146a, 382-3p, 687, 742, 767, 1224 and 1954 *in vivo*. All of these miRNAs were downregulated both *in vivo* and *in vitro*. DOX-induced downregulation of miR-146a has also been observed in cardiomyocytes derived from human pluripotent stem cells.[49] Furthermore, the reduced expression of miR-9-3p, 687 or 1224 has been reported to protect against reactive oxygen species (ROS)-induced apoptosis/cytotoxicity in human and mouse cells *in vitro*,[50–52] and may offer similar protection to Leydig cells following treatment with DOX (also known to generate ROS). The remaining (uncorrelated) DOX-induced *in vivo* miRNA changes could originate from other cell types not investigated here, including other germ cells such as spermatocytes, round spermatids and elongated spermatids, or other testicular cells including the peritubular myoid and blood-, lymphatic- and membrane-related cells.

The *in vitro* approach employed in this study has enabled further exploration of the potential roles for miRNAs within different cell types during DOX-induced testicular toxicity, successfully highlighting key miRNAs, pathways and cell types of interest. These putative mechanisms require further investigation *in vitro*, beginning with experimental validation of specific miRNA and mRNA targets within each cell line and ultimately the development of a co-culture system to examine germ cell and Sertoli cell communications. Thus, while single monolayers are very different from the *in vivo* testis, they do provide cost-effective and time-efficient opportunities to investigate potential mechanisms, examine specific end points of toxicity and identify potential biomarkers of toxicity.

Conclusions

miRNAs play an important role in both normal testicular function and DOX-induced testicular toxicity. Here we have built upon our previous *in vivo* study to show that DOX-induced testicular toxicity involves miRNA changes in all of the three main types of testicular cell (germ, Sertoli and Leydig cells), particularly miRNAs involved in specialised signalling at germ–Sertoli and Sertoli–Sertoli cell junctions. Therefore, it is not only direct DOX-induced toxicity that leads to germ cell loss *in vivo*. miRNA changes in the support (Leydig and Sertoli) cells, and possibly even more distant tissues such as the epididymis, are also important. An improved understanding of mechanisms underlying such testicular toxicity may help to develop biomarkers of testicular toxicity and batteries of simple, end-point specific, cost effective and high-throughput *in vitro* assays for screening the potential toxicity of drugs/chemicals during the compound discovery process.

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgements

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