



Potential of sperm small non-coding RNAs as biomarkers of testicular toxicity in a doxorubicin-induced mouse model

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ABSTRACT

Testicular toxicity is a major concern in cancer chemotherapy and drug development as it can result in infertility; however, there are no effective biomarkers for this adverse effect. To identify new biomarkers, we investigated the expression of small non-coding RNAs (sncRNAs) in a mouse model of doxorubicin (DXR)-induced testicular toxicity. First, we performed small RNA-seq analysis of sperm from DXR-treated or control mice and observed differential expression of many genome-derived sequences. We then performed real-time RT-PCR validation of these sequences and discovered that sncRNA detected by one primers, dxRN_3, showed similar differential expression as that seen in the RNA-seq experiment. These findings suggest that the sncRNAs present in sperm have potential as clinically acceptable biomarkers for testicular toxicity.

1. Introduction

Testicular toxicity is a frequent adverse effect of chemotherapy. Many anticancer drugs attack testicular cells and cancer cells, often resulting in irreversible male infertility [1,2]. Since a patient's fertility after treatment is a key determinant of quality of life, it is important to establish a dosing protocol that kills cancer cells while minimizing toxicity against normal cells [3]. Similarly, predicting the potential toxicity of a candidate substance on the testes is a major concern during drug development [4]. The proposed biomarker candidates for testicular toxicity, such as blood hormone levels or semen examination, have low sensitivity and thus have poor applicability [5]. Therefore, there is a need to develop effective biomarkers for testicular toxicity for use in preclinical and clinical fields.

Non-coding RNAs (ncRNAs) are characterized by a lack of capacity to code for proteins [6]. ncRNAs are divided into two subclasses according to their length: small non-coding RNAs (sncRNAs; less than 200 nt) and long non-coding RNAs (lncRNAs; more than 200 nt). These RNAs were once regarded as "junk sequences"; however, several studies have revealed that they are involved in multiple biological processes, such as cell differentiation, proliferation, and tumorigenesis [7–9]. Recent studies have revealed that sperm also contain a small amount of various sncRNAs, such as microRNA (miRNA), PIWI-interacting RNA (piRNA), and tRNA-derived small RNAs (tsRNAs), which may be involved in post-fertilization phenomena, such as embryonic development and

epigenetic transgenerational inheritance [10–12]. Notably, some miRNAs have been reported to display characteristic changes in expression in a range of diseases, including various types of cancer, Alzheimer's disease, epilepsy, and liver injury [13–16]. sncRNAs show great promise as molecular biomarkers because they can be easily quantified using various widely used standard techniques, such as reverse transcriptase polymerase chain reaction (RT-PCR). As sperm RNA content may be affected by testicular toxicity, the examination of such RNAs may reveal potentially useful non-invasive molecular biomarkers.

The aim of the present study was to investigate the expression profile of sperm sncRNAs in a mouse model of testicular toxicity and to validate differentially expressed RNAs for their use as potential biomarkers. To this end, we employed a mouse model of doxorubicin (DXR)-induced testicular toxicity described in our previous study [17]. DXR is an anthracycline antibiotic and anticancer agent that is commonly used in the treatment of various cancers and causes testicular toxicity at low concentrations [18]. First, we used RNA-seq to perform a comprehensive analysis of the RNA profile in sperm from a mouse model of DXR-induced testicular toxicity. We then used real-time RT-PCR to validate the practical utility of RNAs that showed significant differential expression in the RNA-seq analysis.

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2. Materials and methods

2.1. Animals and chemicals

Male C57Bl/6 N mice were purchased from SLC (Shizuoka, Japan) and maintained in a temperature- and humidity-controlled room with a 12-h light/dark cycle and free access to food and water.

DXR was purchased from Tocris Bioscience (Bristol, UK).

All animal care and experimental procedures were conducted in accordance with the regulations for animal experiments and related activities at Tohoku University. This study was approved by the Tohoku University Institutional Animal Care and Use Committee.

2.2. Treatment protocols

The treatment protocol was based on one described in a previous study [17]. Briefly, the animals were divided into control and DXR-treated groups. The control group received the drug vehicle (saline), and the treated group received 0.2 mg/kg DXR. From 8 to 13 weeks of age, DXR and saline were administered via intraperitoneal injection twice weekly for 5 weeks. Four days after the final dose, the animals were anesthetized and euthanized. Testes and epididymides were collected from both groups and used for subsequent experiments.

2.3. Histopathological examination of testes

Testes from six or seven mice per group were fixed in a methanol:chloroform:acetic acid (6:3:1) solution, treated with 100% ethanol and xylene, embedded in paraffin, and then sectioned into 8- μ m-thick slices. Sections were mounted on glass slides for hematoxylin and eosin staining. Briefly, the sections were deparaffinized with xylene, rehydrated with ethanol, rinsed with distilled water, and stained with hematoxylin solution to visualize the nuclei. Sections were then rinsed with water and stained with eosin to visualize the cytoplasm. The stained sections were observed using a BX63 optical microscope and analyzed using cellSens software (Olympus, Tokyo, Japan) to screen for features of testicular toxicity, such as seminiferous tubules with multinucleated giant cells, vacuolar degeneration, thinning of seminiferous epithelia, and loss of spermatogenic cells.

2.4. Mouse sperm collection

The cauda epididymides were collected from each group, cut using micro-spring scissors and squeezed to extract sperm suspensions, which were then transferred to 1 mL of human tubal fluid medium (101.6 mM NaCl, 4.7 mM KCl, 0.37 mM K_2PO_4 , 0.2 mM $MgSO_4 \cdot 7H_2O$, 2 mM $CaCl_2$, 25 mM $NaHCO_3$, 2.78 mM glucose, 0.33 mM sodium pyruvate, 21.4 mM sodium lactate, 286 mg/L penicillin G, and 228 mg/L streptomycin) [19]. After incubation for 90 min at 37 °C under 5% CO_2 in humidified air, the upper layer of the medium containing motile sperm was collected. The sperm were pelleted by centrifugation and washed twice with PBS. After washing, the pelleted sperm were stored at -80 °C until RNA extraction.

2.5. Sperm RNA extraction

Sperm total RNA was prepared from each group using the method described by Sharma et al. and Danson et al. [12,20]. Briefly, pelleted sperm were suspended in somatic cell lysis buffer (0.1% SDS and 0.5% Triton X-100) for 30 min on ice to eliminate somatic cell contamination. The sperm were then pelleted again by centrifugation. After removing the supernatant, 50 μ L of sperm lysis buffer (6.4 M Guanidine hydrochloride, 5% Tween 20, 5% Triton X-100, 120 mM EDTA, 120 mM Tris, 10 mM DDT, and 10% Proteinase K [20 mg/mL] in ddH_2O) and 50 μ L ddH_2O was added and incubated at 60 °C for 30 min with gentle agitation on a rotator. After incubation, 100 μ L of ddH_2O and 700 μ L of

Qiazol Lysis reagent (Qiagen, Hilden, Germany) were added and vortexed for 10 min. Chloroform (140 μ L) was then added, and the suspension was mixed and centrifuged at 12,000 \times g at 4 °C for 15 min. The upper aqueous phase was then transferred to a new reaction tube and mixed with an equal volume of 70% ethanol. Thereafter, the samples were transferred to spin columns provided with the miRNeasy Mini Kit (Qiagen), and total RNA was eluted according to the manufacturer's protocol. RNA was quantified using a Qubit™ microRNA assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

2.6. Sperm small RNA-seq and data analysis

Sequencing libraries were prepared using sperm total RNA and a SMARTer smRNA-Seq Kit for Illumina (Takara, Shiga, Japan) according to the manufacturer's protocol. Because of the small amount of sperm RNA that could be isolated from a single mouse, RNA from four individuals was pooled to form one sample. Single-end reads (51 bp) were sequenced using the TruSeq Rapid SBS kit (Illumina, San Diego, CA, USA) or TruSeq SBS kit v4 (Illumina) on a HiSeq 2500 instrument (Illumina). The resulting raw reads were then quality checked using the FastQC v0.11.7 program [21]. Adapter trimming was performed using the Cutadapt 1.16 program [22]. In this process, reads below 17 bp in read length after adapter trimming or with one or more 'N' base in the read were filtered out. To annotate these data, the trimmed reads were mapped to the mouse reference genome mm10, miRBase [23], rRNA database (obtained from NCBI), GtRNAdb [24], piRBase [25], Ensembl [26], and Rfam [27] using SPORTS1.0 pipeline [28]. Similarly, we used the HISAT2 [29]-StringTie [30]-edgeR [31] pipeline to detect differentially expressed RNAs between the DXR-treated and control groups. Briefly, the trimmed reads were mapped against mm10 using HISAT2, followed by annotation and transcript quantification using StringTie. Comparison of the transcript expression levels to detect differentially expressed RNAs was performed using edgeR. The detected gene sets with variable expression ($P < 0.05$) were subjected to pathway analysis by Ingenuity Pathway Analysis (IPA) software (version 68752261, Qiagen). The sequence data were deposited in the DDBJ Sequenced Read Archive under accession number DRA012373.

2.7. Real-time RT-PCR

We validated the results of RNA-seq by real-time RT-PCR on RNA extracted from both groups. Because of the small amount of sperm RNA obtained from one mouse, RNA from five animals was pooled to form one sample in this experiment. cDNA synthesis from each RNA sample was performed using the Mir-X miRNA First-Strand Synthesis kit (Takara Bio USA, Inc., Mountain View, CA, USA) according to the manufacturer's protocol. Quantitative real-time PCR was performed using a Takara Thermal Cycler Dice TP960 system (Takara Bio, Inc. Shiga, Japan) to amplify and quantify the small RNAs showing large expression changes between the groups in the RNA-seq experiments. The targets of real-time PCR were selected from small RNAs that showed $|\log_2FC| > 5$ and $FDR < 0.05$ in the sequencing results. Each primer was tested for amplification efficiency; primers with an approximate amplification efficiency of 90–110% were used in the experiments. cDNA was amplified for 40 cycles of denaturation for 5 s at 95 °C and annealed for 20 s at 60 °C. The expression of each small RNA was quantified using the $2^{-\Delta\Delta Ct}$ method. Because a standard internal control gene for PCR of mouse sperm cDNA has not been established, we selected two sequences from the RNA-seq results which exhibited only small differences between the groups ($|\log_2FC| < 1$) and a high expression level ($\log_2CPM > 10$). We then designed primers for these regions and used them as endogenous control genes. The average Ct values obtained from these two primers were used to normalize the expression of the target small RNAs. Independent of this experiment, we performed a similar PCR experiment using U6 small nuclear RNA (snRNA), which is a commonly used endogenous control gene for miRNAs. The primer sequences used

in this experiment are shown in Table 2.

2.8. Statistical analysis

Data are presented as the mean \pm standard deviation (SD). Statistical analysis was performed using Student's *t*-test or Mann–Whitney *U* test in R (<http://www.R-project.org/>). Statistical significance was set at $P < 0.05$. For the small RNA-seq experiment, the exactTest function of the edgeR package was used for comparisons between the groups.

3. Results

3.1. Testicular toxicity assessment

At the end of the treatment, the animals appeared normal, and no deaths occurred in either group. Histopathological examination of testes tissue confirmed that the control group possessed normal seminiferous tubules composed of mature spermatids and sperm cells, with few morphological abnormalities (such as multinucleated giant cells, vacuolar degeneration, thinning of epithelia, and loss of spermatogenic cells) (Fig. 1A and B). In the DXR-treated group, although spermatogenesis was observed in the testes, some tubules showed impairments such as atrophic tubules or vacuolar degeneration (Fig. 1C and D). Correspondingly, although there was no difference in final body weight between the groups (Fig. 1E), the testicular weight of DXR-treated animals was significantly lower than that of the control group ($P < 0.01$, Fig. 1F).

3.2. Sperm small RNA profiling by RNA-seq analysis

To identify small RNAs that could serve as biomarkers for testicular toxicity, we performed RNA-sequencing analysis on sperm extracted from DXR-treated and control mice. First, we conducted rRNA profiling using the SPORTS1.0 pipeline to ensure the extracted RNA was correctly derived from the sperm. Fig. 2A shows that the expression patterns of rRNA precursors in our RNA-seq data were relatively similar to the sequencing results reported by Yang et al. for mouse sperm RNA [32] (accession GSM2304822). As the expression patterns of rRNA precursors have been reported to be cell type-specific [28], the results of our rRNA

analysis suggest that the RNA samples obtained in this study were indeed of sperm origin. Next, we examined the RNA composition of each sample by mapping the reads to various databases (Fig. 2B). miRNAs are considered desirable candidate biomarkers because their functions and potential use as biomarkers have been well studied. However, the sperm RNA obtained in this study contained only a small number ($<2\%$ of all reads) of miRNAs and consisted mostly ($>80\%$) of rRNA-derived or other RNAs (unmapped reads or genome-derived reads). Additionally, we extracted the differentially expressed genes between the groups from these genome-derived reads data and performed pathway analysis using IPA software. We searched for pathways corresponding to the IPA Tox List and found that four pathways, including NRF2-mediated oxidative stress response, were significantly related (Table 1).

3.3. Biomarker candidate selection and real-time PCR validation

Since there were few miRNAs in sperm, we searched for candidate biomarkers based on read mapping to the mouse reference genome mm10. A comparison between the groups of read-accumulating regions identified by *de novo* assembly shows that there are both up- and down-regulated RNAs in sperm from the DXR-treated mice compared to that from the control group (Fig. 3A). We designed primers against regions which demonstrated large differences between the groups and selected seven based on their amplification efficiency, which we named dxRN_1–7 (Table 2). Next, we performed real-time RT-PCR using these primers on sperm RNA from both groups to determine whether the RNA-seq results were reproducible. As a standard internal control gene for PCR of mouse sperm has not yet been established, we performed two independent PCR experiments using two normalization factors: (1) an RNA-seq-based internal control (dxRef_1 and 2) and (2) U6 snRNA (Table 2). In both experiments, only the RNA targeted by the dxRN_3 primer set showed a significant decrease in expression, similar to the RNA-seq results (Fig. 3B).

4. Discussion

In this study, we demonstrated that the expression of sperm sncRNAs was altered under conditions of DXR-induced testicular toxicity and at

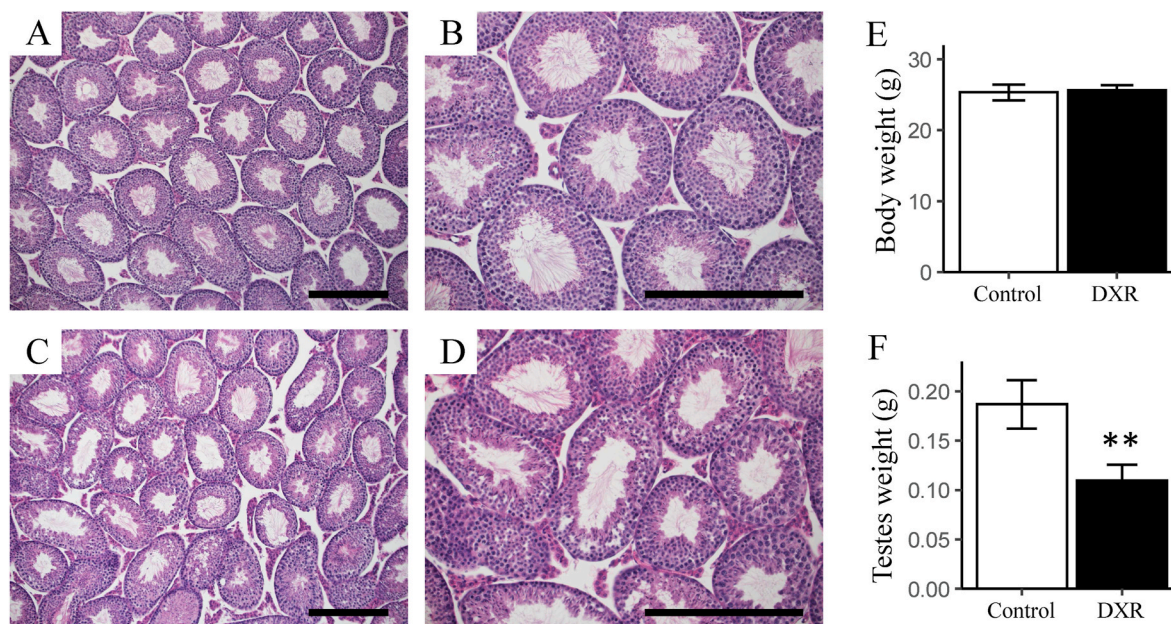


Fig. 1. Assessment of testicular toxicity by hematoxylin and eosin staining, and final body and testes weights. The control group (A and B) showed normal seminiferous epithelium and few morphological abnormalities. In contrast, tubules with reduced seminiferous epithelia and vacuolar degeneration were observed in the DXR-treated group (C and D). Scale bars, 200 μ m. There was no significant difference in body weight between the two groups (E). The testicular weight of DXR-treated testes was significantly lower than the control testes (F). Data are shown as mean \pm SD (**: $P < 0.01$, $n = 6$ (Control group) or 7 (DXR group)).

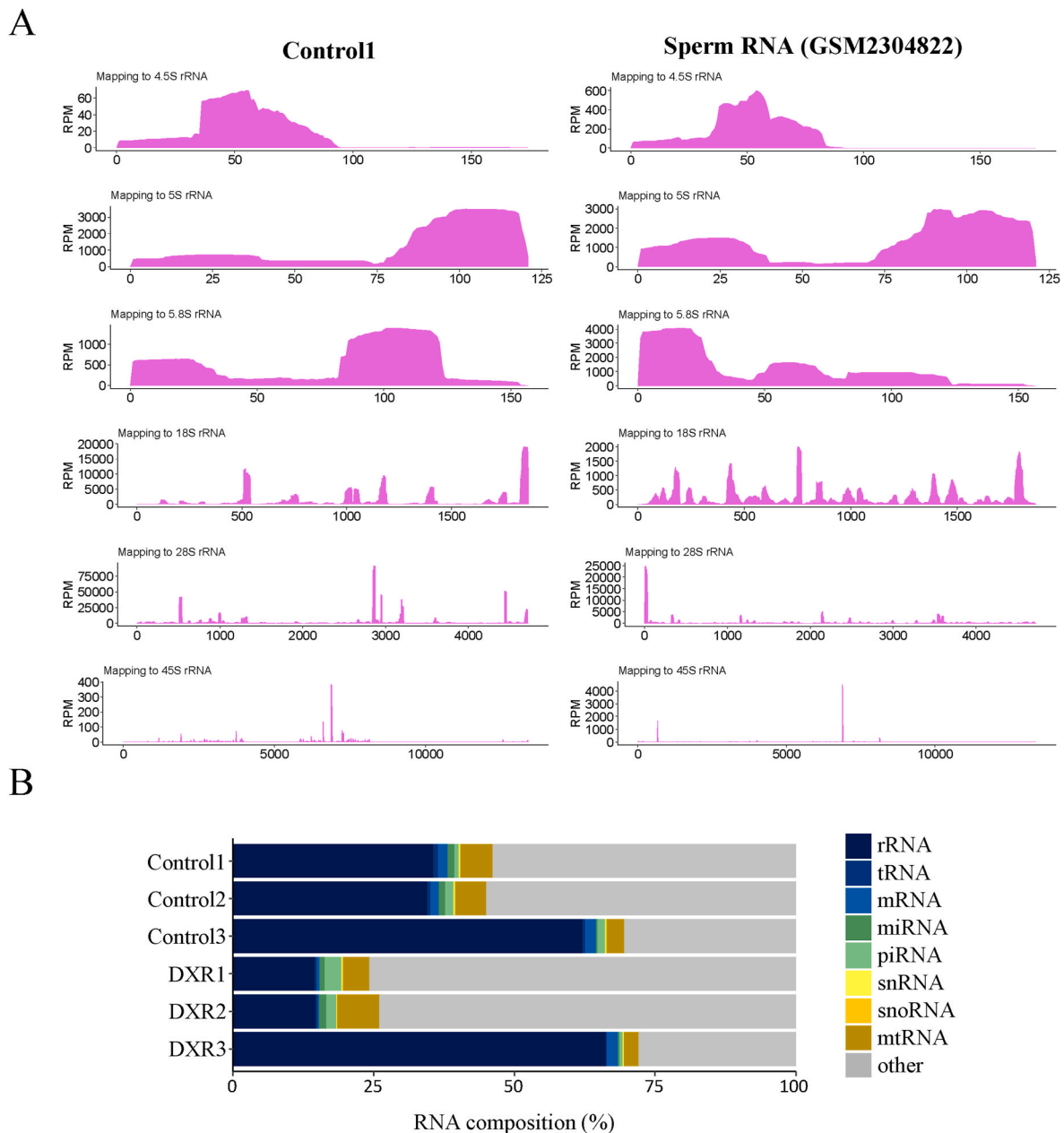


Fig. 2. Sperm small RNA profiling using RNA-seq analysis. (A) The expression patterns of rRNA precursor subtypes. “Control1” is a representative of our RNA-seq data (left panel). The right panel represents the mouse sperm RNA sequencing results reported by Yang et al. (accession GSM2304822). RPM, reads per million clean reads. (B) Classification of all small RNA reads in each group.

least one snRNA was detectable as a biomarker in RT-PCR analysis. Although testicular toxicity is a frequent adverse effect of various drugs, including anticancer agents, there are currently no effective biomarkers to monitor the integrity of the testes [4,33]. The development of effective, non-invasive biomarkers could facilitate the selection of personalized therapeutics or the detection of potential toxicity of chemicals. Therefore, our RNA-seq analysis of DXR-treated mouse sperm may provide clinically useful information.

Recently, epigenetic factors such as DNA methylation and microRNAs have been used as biomarkers in various diseases [34]. Epigenetic biomarkers have been reported in various cancers [35], liver injury [16], and Alzheimer’s disease [14,36], confirming the role of epigenetic regulation in diverse biological phenomena. Previously, we demonstrated that sperm DNA methylation is reduced in DXR-induced testicular toxicity [17]. However, this methylation change was not large enough to be detected by a simple methylation detection method such as

methylation-specific PCR. Since quantitative methylation analysis using next-generation sequencing is precise but costly, more easily detectable candidate biomarkers are required. Sperm snRNAs — the focus of considerable research in recent years — are ideal biomarkers because they are easily detectable using inexpensive methods. Our findings on changes in sperm RNA expression under conditions of testicular toxicity provide useful information for biomarker studies in this context. Although several studies have reported that drug-induced testicular toxicity alters snRNA expression in the testis or sperm [37,38], to the best of our knowledge, this is the first report to show PCR-detectable changes in sperm snRNAs in mice. In addition, our gene pathway analysis using genome-derived reads showed that the changes in sperm RNA content under DXR-induced testicular toxicity are most associated with the NRF2-mediated oxidative stress response pathway. Since oxidative stress is the cause of testicular toxicity in many cases, including DXR-induced testicular toxicity [39,40], it is likely oxidative

Table 1
Top Tox Lists predicted by Ingenuity Pathway Analysis (IPA).

Pathway name	P-value	Genes upregulated in DXR group	Genes downregulated in DXR group
NRF2-mediated Oxidative Stress Response	2.50E-03	DNAJB6, KEAP1, SCARB1	DNAJA1, DNAJB13, DNAJC1, DNAJC11, FTH1, GSTO1, HERPUD1, MAP3K1, PIK3C2G, PIK3R3, PRKCZ, SOD1, STIP1
Cell Cycle: G1/S Checkpoint Regulation	1.67E-02	PAK1IP1	CCND3, CUL1, PA2G4, RBL2, SIN3A
Mechanism of Gene Regulation by Peroxisome Proliferations via PPAR	2.50E-02	NFKBIB, RXRA, NOS2	INSR, PDGFA, PRKAR2B, TNFRSF1A
TR/RXR Activation	4.16E-02	ENO1, RXRA, SCARB1	GPS2, PIK3C2G, PIK3R3

The table shows toxicity-related biological processes such as adaptive, protective, and reparative response to xenobiotic insult predicted by IPA. We inputted 851 genes that were significantly ($P < 0.05$) changed in the DXR group.

stress altered RNA expression in the testis and these RNAs were loaded into sperm. Since down-regulation of NRF2 in testis was reported in other cases of testicular toxicity [41], it is possible that our results can be applied to various testicular toxicities.

However, this study did have some limitations. First, the changes in sncRNA expression observed in our RT-PCR analyses were not large enough to justify their use as biomarkers. Although the sncRNA targeted by dxRN_3 showed a statistically significant change in expression using RT-PCR, the difference was not as high as that demonstrated in the RNA-seq results, and it needs careful discussion before conducting next-phase research. The sncRNA targeted by dxRN_3 is an RNA fragment derived from the Gametogenetin gene (Ggn), which is highly expressed in testes and conserved in humans [42]. Therefore, one method to identify the more appropriate biomarker candidate is to examine the flanking sequences of Ggn or examine Ggn-related biological pathways to understand how this RNA was reduced by DXR and how it was loaded into the sperm. Alternatively, if we can identify more sncRNAs whose expression changes under testicular toxicity, like dxRN_3-recognized sncRNA, we can enhance the power of detection as a biomarker by evaluating their total change. Second, we were unable to examine miRNAs because of their low expression levels. Considering the practical aspects of biomarker applications, miRNAs are desirable biomarkers because of

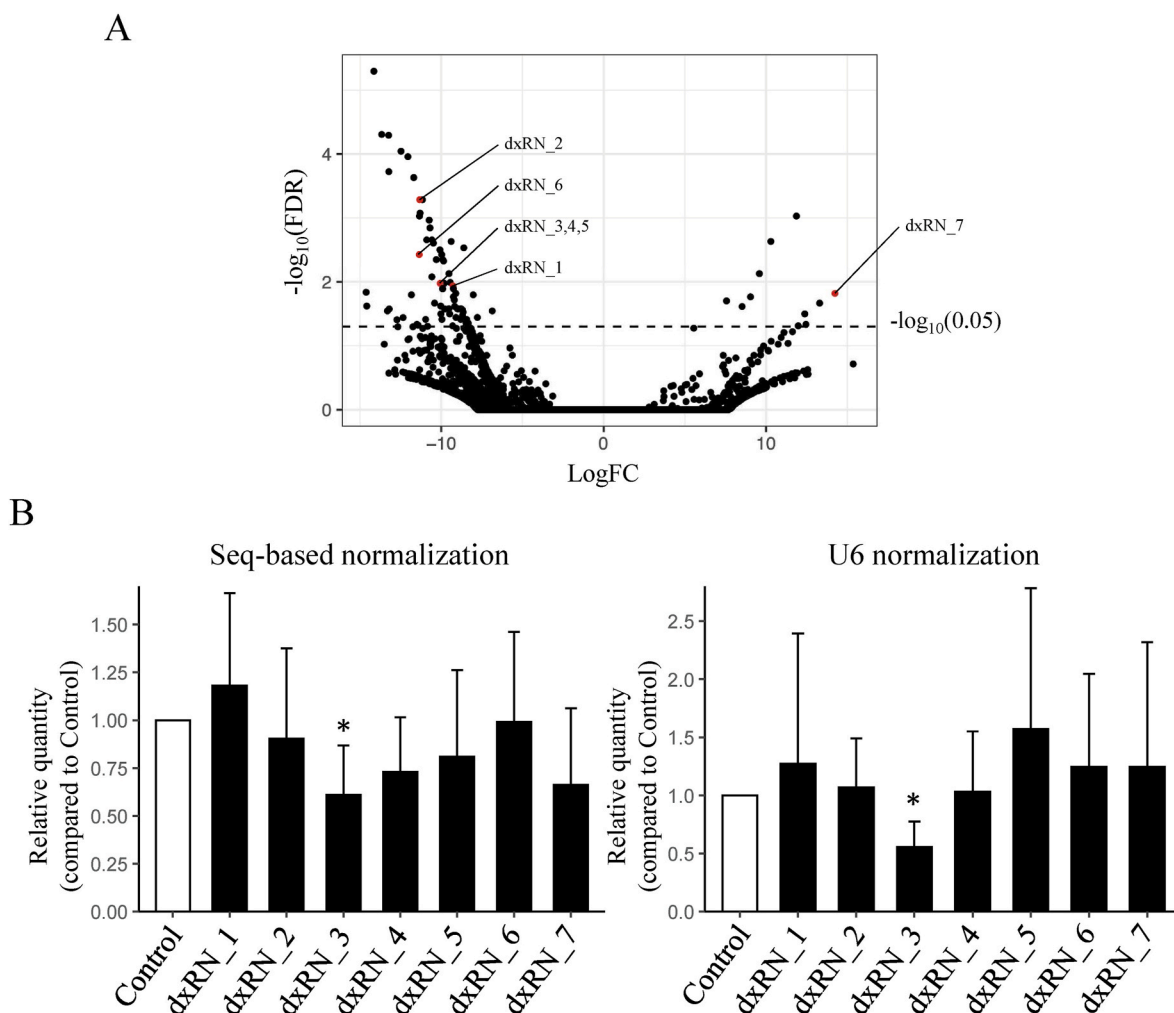


Fig. 3. Biomarker candidate selection and real-time RT-PCR. (A) Volcano plot showing differentially expressed sperm RNAs mapped to mm10 between the groups. The negative \log_{10} -transformed FDR values are plotted against the \log_2 fold change in RNA expression. The RNAs that were selected for subsequent RT-PCR validation as biomarker candidates are plotted in red. (B) The expression of each biomarker candidate RNA was quantified using real-time RT-PCR. Data are shown as expression relative to controls. The data were normalized using the $2^{-\Delta\Delta C_t}$ method, using two approaches for normalization: the graph on the left was normalized by the specific reads selected on the RNA-seq data (dxRef_1 and 2), and the graph on the right was normalized by U6 snRNA. Data are shown as mean \pm SD ($n = 5$, $*: P < 0.05$).

Table 2
Primers used in this study.

Primer name	Primer sequences (5'-3')	Target location	RNA-seq result
Primers for biomarker candidate sperm small RNA			
dxRN_1	CCAAGTCCAGTTGCTCTGG	chr6	Down-regulated
dxRN_2	AGGTAGTGAGGGGAGGAG	chr7	Down-regulated
dxRN_3	CCTAGATCTCGGGGTCGTC	chr7	Down-regulated
dxRN_4	GATCCAAGGGATCAGGGTTGG	chr7	Down-regulated
dxRN_5	TACTTCCGAGTCTGGCCTGA	chr7	Down-regulated
dxRN_6	GACGCCGTAAGATGCTTTC	chr7	Down-regulated
dxRN_7	GAAGTGGTGGTCACTGTCCC	chr9	Up-regulated
Primers for endogenous control gene			
dxRef_1	GCTGGAGTGCAGTGGCTATT	chr12	–
dxRef_2	CTATGTTGCCAGGCTGGAG	chr12	–
U6 Fw	Commercial primer	–	–
U6 Rv	Commercial primer	–	–

All primers listed in the table except U6 primers were used as forward primers. The reverse primer was a commercially supplied universal primer in Mir-X miRNA qRT-PCR TB Green kits (Takara Bio). The primers for U6 were also included in the kit.

their high interspecies conservation and well-understood molecular mechanisms [43]. This is a limitation of this model rather than a limitation of the research, and it is expected that large amounts of samples can be collected when using human sperm samples instead of mouse samples; therefore, this problem will be resolved in the development of clinical biomarkers. In a future study, we plan to screen larger amounts of sperm RNAs and identify miRNA sequences that show more pronounced changes.

In summary, this study demonstrated that sncRNAs in sperm are differentially expressed at levels detectable by RT-PCR in mouse models of DXR-induced testicular toxicity. Although the results of this study are insufficient for application as a practical biomarker, they indicate that sperm RNAs may yield promising biomarkers for testicular toxicity.

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Data availability

The data that support the findings of this study are available at the DDBJ Sequenced Read Archive under accession number DRA012373.

Declaration of competing interest

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