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Genomic and proteomic analyses of 1,3-dinitrobenzene-induced testicular toxicity in Sprague–Dawley rats

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ABSTRACT

1,3-Dinitrobenzene (DNB) is an industrial intermediate and testicular toxicant that has been shown to target Sertoli cells. The mechanism of action of DNB in the testis, however, is unclear. To investigate global alterations in gene or protein expression during testicular toxicity, testes from rats treated orally with DNB were subjected to microarray and two-dimensional gel electrophoresis (2-DE) analyses. Histopathological abnormalities were detected in the testes of the DNB-treated rats. Microarray analysis revealed that, during early testicular toxicity, several genes involved in apoptosis, germ cell/Sertoli cell junction, and tight junction signaling pathways were differentially expressed. Based on 2-DE analysis, 36 protein spots showing significantly different expression during early testicular toxicity were selected and identified. Network analysis of the identified proteins revealed that these proteins are associated with cellular development or reproductive system diseases. Collectively, these data will help clarify the molecular mechanism underlying testicular toxicity in DNB-exposed rats.

1. Introduction

Male reproductive toxicity has been a major concern in recent years because of rising levels of environmental toxicants or pharmaceuticals, including pesticides, pollutants, food additives, and metals. It has been reported that various compounds, such as 2,5-hexanedione and benzimidazole fungicides, induce testicular toxicity by interfering with the structural assembly and function of seminiferous tubule cells [1,2]. Additionally, many testicular toxicants have been reported to disrupt spermatogenesis by altering the functioning of Leydig, Sertoli, and germ cells [3–6]. Sertoli cells promote germ cell development by providing a specialized morphological and biochemical environment. Defects in Sertoli cell function can cause impaired spermatogenesis and male infertility, and several toxicants have been implicated in this process, including the drugs cisplatin and gossypol, plasticizers, pesticides, and organic solvents [7–9].

1,3-Dinitrobenzene (DNB), an industrial intermediate chemical, is known to induce testicular toxicity by damaging Sertoli cells [10]. DNB is a nitroaromatic compound used in the synthesis of dyes, plastics manufacturing industry and as explosives. Humans can be exposed to DNB by contaminated drinking water, food, air, or soil. In the rat model, the half-life of DNB was estimated to be approximately 10 h and was shown to induce neurotoxicity [11]. DNB has also been reported to induce testicular toxicity by damaging Sertoli cells [12]. Histopathological analyses of DNB-treated rodents have shown injury to seminiferous tubules, with multinucleated cells and Sertoli cell vacuolization [10,13]. It has been reported that testicular toxicity or male infertility caused by DNB is the result of damage to Sertoli cells or surrounding germ cells [14,15]. Several studies have attempted to unravel the mechanism by which DNB functions both *in vitro* and *in vivo*. Previously, we found that DNB induced apoptosis and G2/M phase cell cycle arrest *via* JNK signaling *in vitro* using TM4 mouse Sertoli cells [16]. In an *in vivo* model, Muguruma et al. (2005) suggested that DNB induced apoptosis *via* the mitochondrial pathway; however, the underlying molecular mechanism remains unclear (*i.e.*, which signaling events lead to the disruption of Sertoli cell function or Sertoli/germ cell interactions).

Recent studies utilizing genomic approaches have provided insight into the molecular mechanism of chemicals known to induce hepatotoxicity and nephrotoxicity [17–20]. Additionally, proteomic technology has been used to identify new biomarkers for diagnostic and prognostic purposes as well as to uncover the action of various toxicants [21–23]. Analyses by microarray and two-dimensional electrophoresis (2-DE) have associated several

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reproductive toxicants, including acrylamide, sulfasalazine, 2,5-hexandione, and xenoestrogens, with male reproductive toxicity [24–28]. For DNB-induced testicular toxicity, changes in the expression of several genes related to the 20S proteasome core subunit or junction proteins, including ZO-1, N-cadherin, and Connexin 43, have been analyzed *in vitro* and *in vivo* [29,30]. However, global gene expression profiles and differential protein expression have not been reported. In the present study, we performed comprehensive microarray and 2-DE analyses using testes isolated from DNB-treated rats. Our results provide molecular insight into DNB-induced testicular toxicity.

2. Materials and methods

2.1. Animal treatments

Eight-week-old Sprague–Dawley rats (specific pathogen-free males) purchased from SLC Co. (Hamamatsu, Japan) were maintained under a 12-h light/dark cycle at a controlled temperature and humidity in an animal room and acclimated at least two weeks prior to the start of the experiments. The rats were fed standard food pellets and water *ad libitum*; 60 rats were randomized to each experimental group ($n = 5$). DNB was purchased from Sigma (D194255; St. Louis, MO, USA) and dissolved in corn oil. The rats were treated daily with oral injections of vehicle or DNB at a dose of 3, 7, or 15 mg/kg for up to three days. The dosage and its duration were determined from preliminary studies that demonstrated testicular toxicity based on histological findings. DNB-treated and time-matched vehicle control rats were sacrificed on days 1, 3, and 7 after final exposure to the chemical. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and conducted in accordance with Association of Assessment and Accreditation of Laboratory Animal Care International guidelines. The rats were sacrificed using isoflurane, and their testes and epididymides were collected, weighed, and frozen. Half of the testes and epididymides were placed in 10% Bouin's solution for histological analysis, while the other half were flash-frozen in liquid nitrogen and stored at −80 °C for microarray and proteomic analyses.

2.2. Organ weight and histopathology

Total body weight and the weights of the testes and epididymides were measured, and the relative testis and epididymis weight ratio was calculated. The mean ± standard deviation (SD) is presented for the relative organ weight ratio. The testes and epididymides were fixed for a maximum of 48 h, embedded in paraffin, cut into 4-μm sections, stained with hematoxylin and eosin (H&E), and analyzed by light microscopy (E400; Nikon, Tokyo, Japan). Three rats chosen from five tested animals based on our histologic data were used for microarray and proteomic analyses.

2.3. Microarray analysis

Testis samples for the 7 mg/kg-treated group at all time points were homogenized in Trizol reagent (Invitrogen, Carlsbad, CA, USA), and isolated total RNA was repurified using an RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The total RNA was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA), while the quality was evaluated using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) for the DNA binding experiments (chromatin immunoprecipitation).

An Affymetrix GeneChip Rat Genome 230 2.0 array containing 31,099 probes was used for the microarray experiment; all processing, including probe labeling, hybridization, washing, and staining, were performed according to the manufacturer's protocol (Affymetrix, Santa Clara, CA, USA). For each array, the data were pre-processed, cell intensity files (CEL) were generated, and the data were analyzed using GenPlex software (Istech Inc., Goyang, Korea). The data were normalized using global scale normalization. Differentially expressed genes were selected based on a >1.3-fold change and Student's *t*-test ($P < 0.05$). The selected genes were analyzed using a hierarchical clustering algorithm; they were imported into Ingenuity Pathways Analysis software (IPA; Ingenuity Systems, Redwood, CA, USA), and the biological functions and molecular networks were analyzed. A network was constructed for each group using the "direct or indirect interaction" algorithm. The selected genes were annotated based on NetAffx (http://www.affymetrix.com).

2.4. Proteomic analysis

Testicular samples collected from the 7 mg/kg-treated group and corresponding controls on day 1 were homogenized in buffer (8 M urea, 2 M thiourea, 2% CHAPS, 20 mM DTT, and 40 mM Tris, pH 8.8) containing protease inhibitor cocktail (Complete Protease Inhibitor Cocktail Tablets; Roche Applied Science, Mannheim, Germany), and centrifuged at 20,400 × *g*-force for 2 h at 4 °C. Tissue lysates were then desalted and further concentrated using a Vivaspin 500 centrifugal concentrator (Vivascience, Hanover, Germany). The protein concentrations were determined using a Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). 2-DE was performed as reported previously [21]. Briefly, 1 mg of total protein from DNB-treated and control testes was applied to immobilized pH 3–10 nonlinear gradient strips (Bio-Rad Laboratories) by gel rehydration. Isoelectric focusing was performed at approximately 150,000 Vh at 20 °C, followed by 2-DE using 12.5% polyacrylamide gels. After protein fixation, the gels were stained with Coomassie Brilliant Blue G250, destained with H_2O, scanned using a Bio-Rad GS710 densitometer (Bio-Rad Laboratories), and converted into electronic files, which were then analyzed using the ImageMaster Platinum 6.0 image analysis program (Amersham, Uppsala, Sweden).

Protein spots that varied by more than two-fold compared with the untreated control were regarded as differentially expressed spots. Proteins were identified by mass fingerprinting or matching with various internal 2-DE maps, and the protein spots were excised from the Coomassie blue-stained gels and digested with trypsin (Promega, Sunnyvale, CA, USA). For matrix-assisted laser desorption/ionization-tandem time of flight (MALDI-TOF/TOF) analysis, mass spectra were acquired on a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA) operating in the mass spectrometry (MS) and tandem mass spectrometry (MS/MS) modes Peptide mass fingerprinting and MS/MS ion search were performed using the Mascot search engine included in the GPS Explorer software package. The protein score defined in Mascot, which uses the probability-based Mowse algorithm, is calculated by $-10 \times \log(P)$, where P is the probability that the observed match for this ion is a random event. Mascot software automatically calculated the score, and manual inspection was used to verify the matched proteins by molecular weight and pI-value consideration. Mass spectra used for manual *de novo* sequencing were annotated using the Data Explorer software (Applied Biosystems).

3. Results

3.1. DNB-induced testicular toxicity

Testicular toxicity was observed in Sprague–Dawley rats following three days of DNB treatment. The relative organ weight

Table 1
Body and organ weights of testes and epididymides after DNB exposure.

Duration	Dosage	Body weight		Testes		epididymides	
		Before administration	After administration	Absolute weight (g)	Relative ratio	Absolute weight (g)	Relative ratio
1 day	Vehicle	325.87 ± 10.28	318.28 ± 17.51	3.26 ± 0.001	1.03 ± 0.05 (100)	0.88 ± 0.06	0.28 ± 0.00 (100)
	3 mg/kg	327.03 ± 6.53	324.13 ± 10.39	3.21 ± 0.79	0.99 ± 0.06 (96)	0.79 ± 0.06	0.24 ± 0.01** (88)↓
	7 mg/kg	327.30 ± 5.07	324.34 ± 12.48	3.41 ± 0.81	1.05 ± 0.06 (103)	0.81 ± 0.15	0.25 ± 0.04 (91)
	15 mg/kg	331.34 ± 2.88	318.45 ± 1.32*↓	2.90 ± 0.72**↓	0.91 ± 0.04* (89)↓	0.72 ± 0.08	0.23 ± 0.02* (82)↓
3 day	Vehicle	321.37 ± 12.09	332.85 ± 7.39	3.29 ± 0.87	0.99 ± 0.10 (100)	0.87 ± 0.09	0.26 ± 0.02 (100)
	3 mg/kg	326.46 ± 6.28	335.97 ± 14.46	3.29 ± 0.87	0.98 ± 0.03 (99)	0.87 ± 0.05	0.26 ± 0.01 (99)
	7 mg/kg	325.34 ± 14.54	324.21 ± 20.62	3.16 ± 0.86	0.98 ± 0.06 (99)	0.86 ± 0.04	0.26 ± 0.02 (101)
	15 mg/kg	330.05 ± 3.11	321.84 ± 11.43	2.52 ± 0.75*↓	0.78 ± 0.02 (79)	0.75 ± 0.08	0.23 ± 0.02 (90)
7 day	Vehicle	330.40 ± 18.18	351.60 ± 20.95	3.38 ± 0.92	0.96 ± 0.03 (100)	0.92 ± 0.06	0.26 ± 0.03 (100)
	3 mg/kg	329.18 ± 9.63	335.66 ± 12.04	3.35 ± 0.91	1.00 ± 0.12 (104)	0.91 ± 0.08	0.27 ± 0.03 (103)
	7 mg/kg	328.50 ± 13.77	323.72 ± 34.51	2.79 ± 0.86	0.87 ± 0.12 (91)	0.86 ± 0.03	0.26 ± 0.04 (100)
	15 mg/kg	327.42 ± 7.65	343.06 ± 13.09	1.90 ± 0.66*↓	0.55 ± 0.04** (58)↓	0.66 ± 0.10**↓	0.19 ± 0.04** (73)↓

Values represent the means ± standard deviation (SD) for five rats in each group. Relative organ weight was calculated using the ratio of the target organ weight to body weight and is represented as a percentage. The relative fold change compared to the corresponding control is shown in parentheses.

* $P < 0.05$ *vs.* corresponding control.

** $P < 0.01$ *vs.* corresponding control. *P*-value was calculated using Student's *t*-test.

ratio of the testes and epididymides to the total body weight was determined (Table 1). In the 3 mg/kg-treated group, there was no significant change in the testes and epididymal weights, while a slight decrease in testes weight was observed on day 7 in the 7 mg/kg-treated group. However, in the 15 mg/kg-treated group, the testes and epididymides weights were decreased significantly at all time points. Histopathological analyses of the testes and epididymides demonstrated tubular atrophy and multinucleated cells in the testes of the DNB-treated rats, with oligospermia, cellular debris (head and tail regions), and multinucleated cells in the epididymides (Fig. 1 and Table 2). These results indicate testicular and epididymal toxicity due to the administration of DNB, and that the severity of these effects was increased according to the dose level and duration after final exposure.

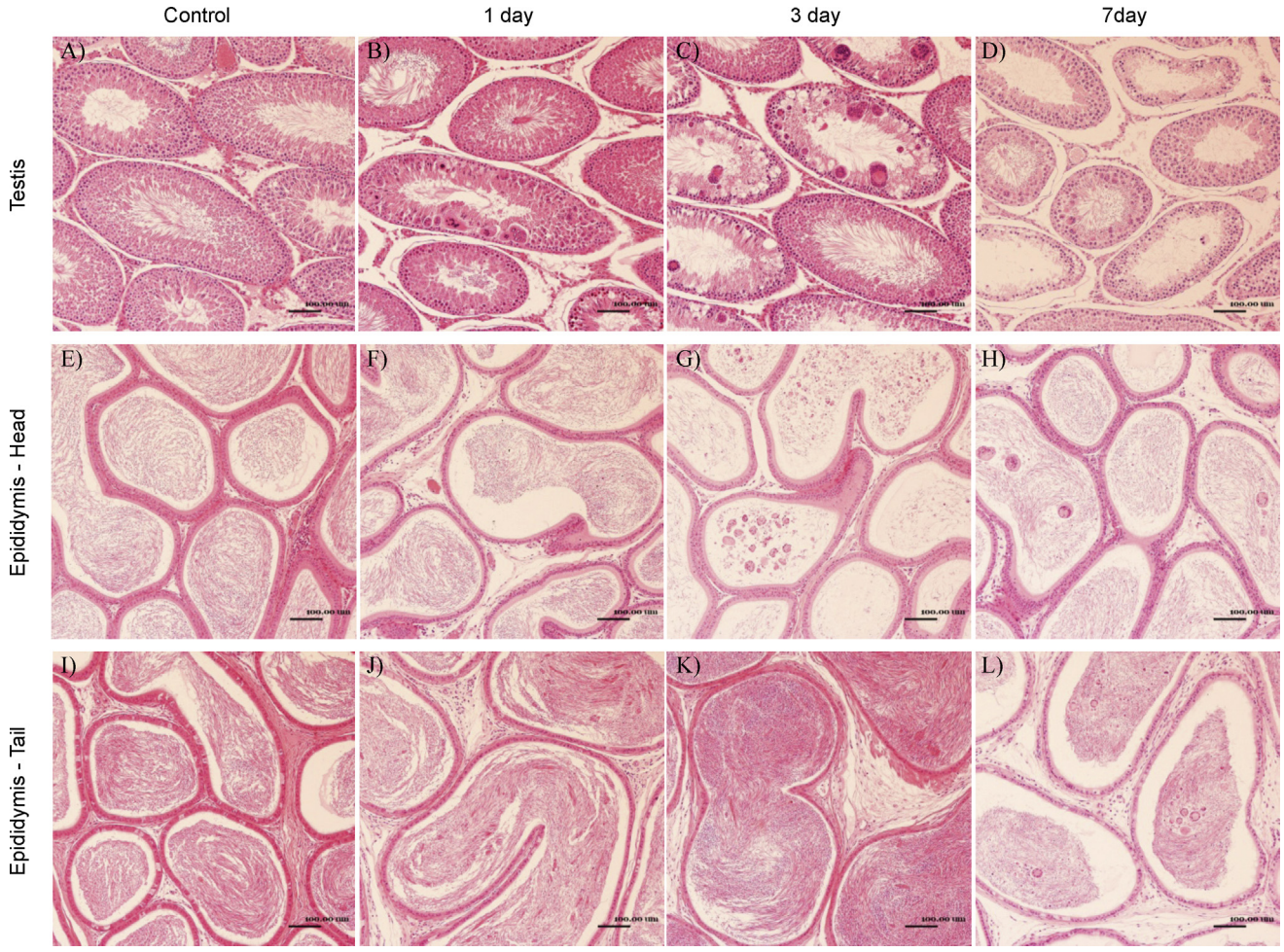

Fig. 1. Light microscopic images of the testis and epididymis in the 7 mg/kg-treated group: (A)–(D) testis; (E)–(H) head of the epididymis; (I)–(L) tail of the epididymis. The scale bar represents 100 μm.

Table 2
Histopathological observation of testes and epididymides after DNB exposure.

Group organ/lesions (no. of animals)	3 mg/kg			7 mg/kg			15 mg/kg		
	1 day (5)	3 day (5)	7 day (5)	1 day (5)	3 day (5)	7 day (5)	1 day (5)	3 day (5)	7 day (5)
Testes									
Tubular atrophy	– (5)	– (5)	– (5)	++ (1)	+ (1)	++ (5)	+++ (4)	+++ (4)	+++ (4)
Multinucleated cells, tubule	– (5)	– (5)	– (5)	+ (2)	+++ (5)	+++ (5)	– (5)	– (5)	+ (3)
Epididymides									
Sperm granuloma	– (5)	– (5)	– (5)	– (5)	+++ (2)	– (5)	– (5)	– (5)	– (5)
Oligospermia, head	– (5)	– (5)	– (5)	– (5)	+ (3)	– (5)	+ (4)	+++ (4)	+++++(4)
Oligospermia, tail	– (5)	– (5)	– (5)	– (5)	– (5)	– (5)	– (5)	– (5)	+ (2)
Cell debris, head	– (5)	– (5)	– (5)	– (5)	+ (5)	+ (3)	+ (4)	+++ (4)	+++ (3)
Cell debris, tail	– (5)	– (5)	– (5)	– (5)	– (5)	+ (3)	– (5)	– (5)	+++ (4)
Multinucleated cells	– (5)	– (5)	– (5)	– (5)	+ (5)	++ (4)	– (5)	– (5)	– (5)

Five rats per group were treated daily with oral injections of vehicle or DNB up to 3 days and were then sacrificed on days 1, 3 and 7 after final exposure to the chemical. Lesion severity is indicated as follows: +, minimum or very slight degree or amount; ++, slight degree or small amount present; +++, moderate, median or middle severity or amount present severe; ++++, marked severity or degree of change, large amount present; +++++, highest degree or severity, maximum amount present. The number of animals representing lesion severity is indicated in parentheses. There were no lesions in the corresponding controls.

3.2. Differentially expressed genes in the testes of DNB-treated Sprague–Dawley rats

To elucidate the molecular mechanism underlying DNB-induced testicular toxicity, testicular gene expression profiles were analyzed using the Affymetrix GeneChip system as described in Section 2. Based on histological observation, we chose the 7 mg/kg treated group for our microarray analysis because the testicular lesions in this group increased gradually over time. Each data set consisted of an expression array from three animals in the treated groups (7 mg/kg; all time points up to day 7) and time-matched controls. For microanalysis, three replicates of each group were used; however, two replicates of the day-1-treated group and corresponding controls were used due to low correlation. In total, 8534 genes, including 1483, 2793, and 5941 genes corresponding to the treated group on day 1, 3, or 7, respectively, were differentially expressed with a >1.3-fold change ($P < 0.05$; two-tailed, unpaired Student's *t*-test) compared with the control. Among them, 580 genes were up-regulated and 903 were down-regulated on day 1; 1231 genes were up-regulated and 1562 were down-regulated on day 3; and 3118 were up-regulated and 2823 were down-regulated on day 7 compared with the corresponding time controls. Of these genes, many with altered expression in the DNB-treated group were not annotated. To further analyze the biofunction and canonical pathways of these genes, we lowered the cutoff condition for selecting differentially expressed genes to a >1.3-fold change ($P < 0.05$). *K*-means clustering revealed that the genes expression patterns could be clustered into time specific and time dependent group, and also discriminated into two main groups, up-regulated (clusters 3 and 7) or down-regulated (clusters 8 and 10) on day 1 and day 3, respectively (Fig. 2A). Genes belonging to clusters 5 and 6 increased or decreased gradually, respectively, according to time after DNB exposure (Fig. 2B). Gradually increased or decreased genes were involved in signaling pathways such as MAPK, T-cell-receptor signaling, Wnt, or calcium signaling (Supplementary Table 1). In *k*-means clustering, genes commonly up- or down-regulated in all three groups were not shown because the gene set was too small. We analyzed the Venn diagram using genes deregulated at each time point, and commonly deregulated genes are shown in Fig. 2C. Genes commonly deregulated in all three groups or in both the 1- and 3-day groups, which can be grouped with early testicular toxicity, are listed in Table 3. Several genes, including *Wnt4*, *Testin* and *Tnfrsf12a* were commonly up-regulated at all-time points in the DNB-treated groups; in comparison, *Gpr61*, *Lbxcor1* and *Gjb6* were commonly down-regulated in the DNB-treated groups. For the specifically expressed genes in the one- and three-day groups, *Cyp46a1*, *Lif*, *Itih4*, and *RICS* were up-regulated while *Phf2*, *Zfp68*, *RGD1309621*, and *Psip1* were down-regulated in the testis during early testicular toxicity.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reprotox.2013.10.004.

3.3. Functional analysis of differentially expressed genes

We analyzed the biofunction and canonical pathways of the differentially expressed genes at each time point in the DNB-treated group using ingenuity pathways analysis (IPA). Biofunction analysis revealed that the altered genes in the one- and three-day groups were mainly involved in cell death, whereas the genes in the seven-day group were involved in DNA replication, recombination, and repair (Table 4). This indicates that DNB triggers testicular cell death during early exposure. Subsequently, in the seven-day group, the testes were severely injured and DNA replication and repair systems were activated to overcome this injury.

As shown in Fig. 3, canonical pathway analysis demonstrated that cancer-related genes, apoptosis, BMP signaling, and the germ cell/Sertoli cell junction signaling pathway were involved in early testicular toxicity (one- and three-day groups), whereas genes involved in mitosis (*e.g.*, Polo-like kinase) and the protein ubiquitination pathway were significantly involved in late testicular toxicity (seven-day group). Cell cycle checkpoint genes and ERK/MAPK signaling were commonly deregulated in the DNB-treated groups. We therefore focused on the gene expression patterns of representative pathways for early and late testicular toxicity. Among nine genes related to apoptosis, pro-apoptotic genes, including *Bak1* (BCL2-antagonist/killer 1), *Apaf1* (apoptotic peptidase activating factor 1), and *Bbc3* (Bcl-2 binding component 3), were up-regulated, whereas the anti-apoptotic gene *Birc2* (baculoviral IAP repeat-containing 2) was down-regulated in early testicular toxicity (Fig. 4A). In total, 29 and 30 genes related to germ cell/Sertoli cell junction and tight junction signaling were differentially expressed in early testicular toxicity, respectively. In particular, genes involved in the interaction between germ and Sertoli cells such as *Cdh2* (cadherin 2), *Ctnna1* (catenin [cadherin-associated protein] alpha 1), *Ctnnb1* (catenin [cadherin associated protein] beta 1), *Itgb1* (integrin beta 1 [fibronectin receptor beta]), and *Sorbs1* (sorbin and SH3 domain containing 1: alias, *Ponsin*) were up-regulated during early testicular toxicity (Fig. 4A). For the tight junction signaling pathway, genes such as *Tiam1* (T-cell lymphoma invasion and metastasis 1), *Jam2* (junction adhesion molecule 2), *Cgn* (cingulin), and *Mylk* (myosin, light polypeptide kinase) were up-regulated, whereas *Palv3* (Poliovirus receptor-related 3: alias, *Nectin*) and *Smurf1* (SMAD specific E3 ubiquitin protein ligase 1) were

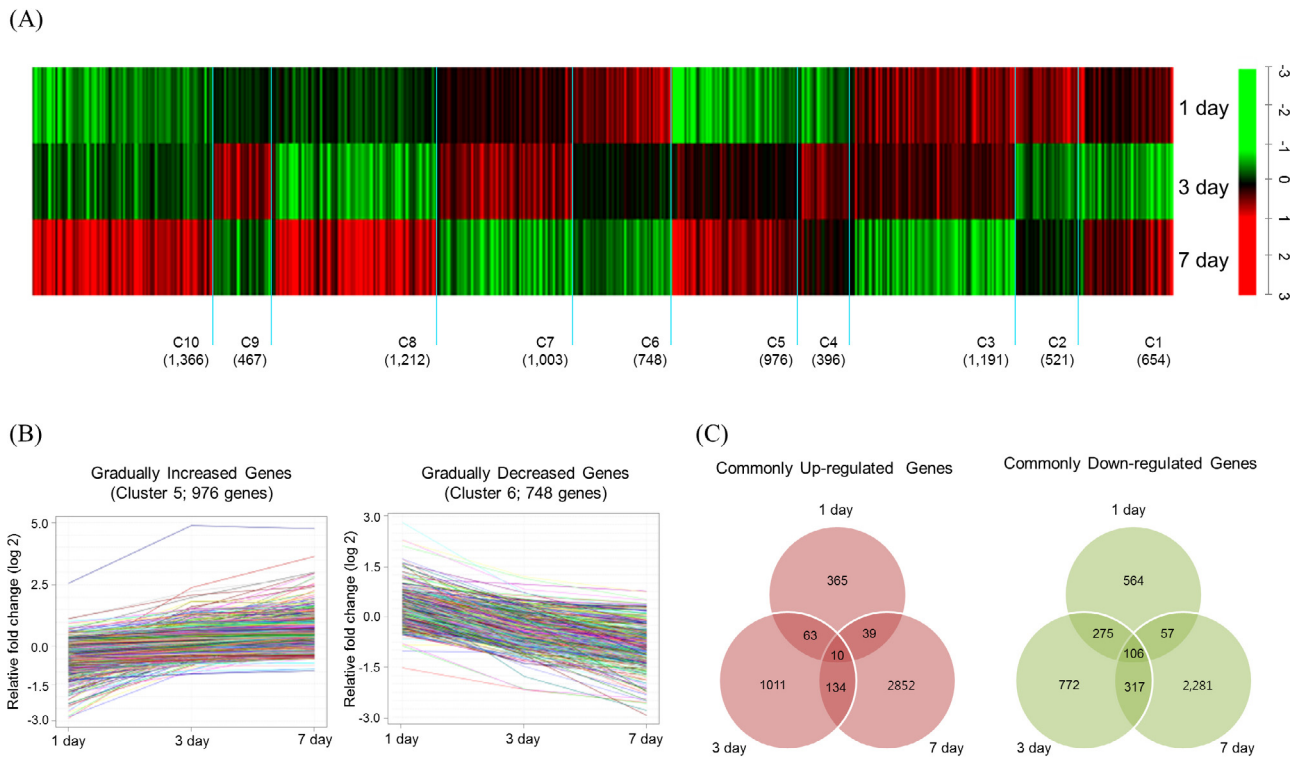

Fig. 2. *K*-means clustering of differentially expressed genes in DNB-treated groups ($k = 10$, total cluster = 10). (A) Ten clusters are highlighted, with colored lines and the number of genes belonging to each cluster represented in parentheses. (B) Expression profiles of genes belonging to clusters 5 and 6, which represent gradually up- or down-regulated genes according to time progression. (C) Venn diagram of commonly up and down-regulated genes at all time points.

down-regulated during early testicular toxicity (Fig. 4A). In the seven-day treated group, 80 genes related to ubiquitination were differentially expressed, and resulted in the worsening of the testicular lesions (Fig. 4B). The gene encoding ubiquitin-activation enzyme (E1 type, *Ube1x*) was down-regulated. In terms of ubiquitin-conjugating enzymes (E2 types), several genes, including *Ube2a*, *Ube2c*, *Ube2d1*, *Ube2n*, and *Ube2m*, were down-regulated while others like *Ube2b*, *Ube2d2*, *Ube2e3*, and *Ube2g1* were up-regulated in the seven-day group. Genes encoding ubiquitin-specific peptidases or proteases were mostly up-regulated in the seven-day group (*Usp28*, *Usp32*, *Usp40*, *Usp47*, *Usp48*, and *Usp54*). Among genes encoding components of the proteasome complex, those encoding proteasome subunit α- and β-types were up-regulated (*Psma3*, *Psma4*, *Psma5*, *Psmb2*, and *Psmb6*), except for *Psmb9*. Additionally, a gene encoding a component of polyubiquitin, ubiquitin D (*Ubd*), was time-dependently down-regulated while ubiquitination factor (*Ube4a*) was up-regulated in the DNB-treated groups.

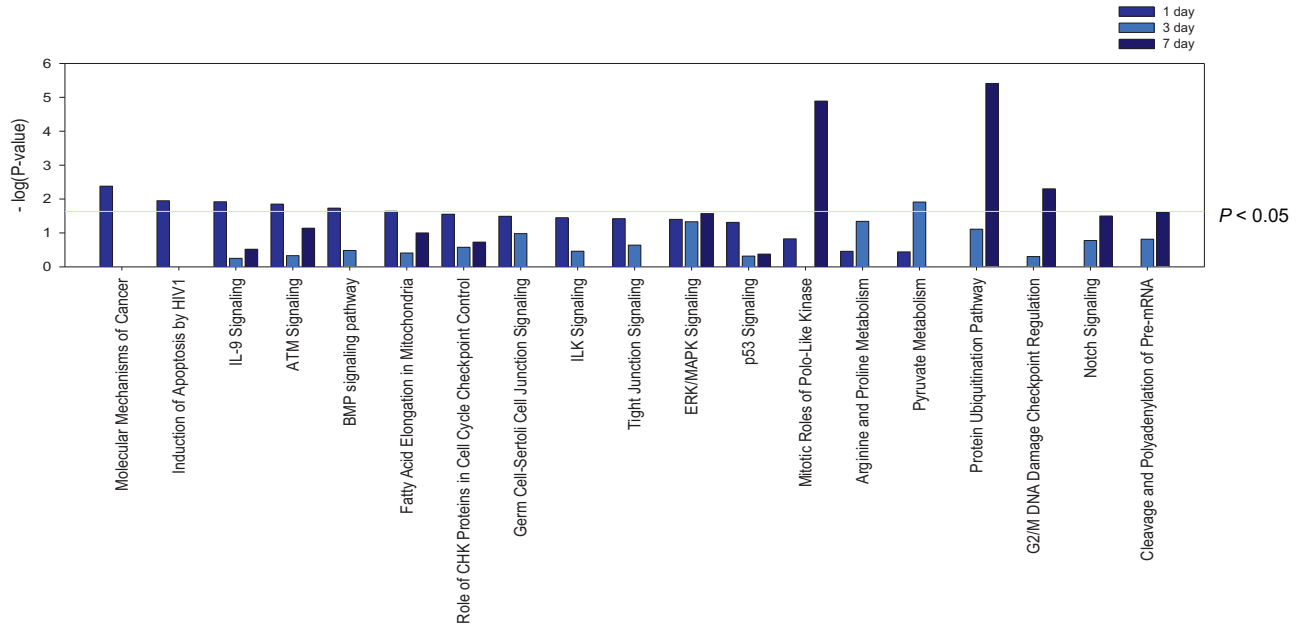

Fig. 3. Canonical pathway analysis of the differentially expressed genes in the DNB-treated groups. Categories matched over statistical significance ($P < 0.05$) in each treated group are depicted. The dotted line indicates the threshold of statistical significance ($P < 0.05$).

Table 3
Differentially expressed genes in the testis after DNB exposure.

Gene_symbol	Gene_title	Acc. no.	Fold-change (Log 2)		
			1 day	3 day	7 day
Commonly regulated genes at all time points in the DNB-treated group					
Wnt4	Wingless-related MMTV integration site 4	NM_053402	2.6	4.9	4.8
Testin	Testin gene	NM_173132	2.1	1.6	3.7
Tnfrsf12a	Tumor necrosis factor receptor superfamily, member 12a	NM_181086	1.0	1.5	1.7
Mte1	Mitochondrial acyl-CoA thioesterase 1	NM_138907	0.9	1.3	1.4
Cish	Cytokine inducible SH2-containing protein	XM_001074104	1.3	3.2	1.3
Fnbp1	Formin binding protein 1	NM_138914	1.0	1.4	1.2
Golph4	Golgi phosphoprotein 4	XM_001062460	1.6	1.5	1.0
Ctgf	Connective tissue growth factor	NM_022266	1.2	1.6	0.9
Ier3	Immediate early response 3	NM_212505	1.0	1.0	0.6
Nes	Nestin	NM_012987	1.1	1.6	0.5
Gpr61	G protein-coupled receptor 61	NM_001107715	-2.6	−4.9	−2.8
Lbxcor1	Ladybird homeobox 1 homolog (*Drosophila*) corepressor 1	XM_001075793	−1.6	−3.7	−2.2
Gjb6	Gap junction membrane channel protein beta 6	NM_053388	−2.3	−1.6	−1.7
Dscr6	Down syndrome critical region homolog 6 (human)	NM_001105892	−1.2	−1.1	−1.7
Pcdh8	Protocadherin 8	NM_022868	−1.5	−1.5	−1.6
Shc3	src homology 2 domain-containing transforming protein C3	NM_001105743	−1.0	−1.4	−1.1
Nol4	Nucleolar protein 4	NM_001107401	−1.4	−1.3	−1.0
Ddx28	DEAD (Asp-Glu-Ala-Asp) box polypeptide 28	NM_001108898	−1.1	−1.4	−1.0
Trmt12	tRNA methyltransferase 12 homolog (*S. cerevisiae*)	XM_001068980	−1.3	−1.1	−1.0
Pitx2	Paired-like homeodomain transcription factor 2	NM_001042505	−1.2	−3.1	−0.9
Specifically expressed genes in the 1 and 3 day DNB-treated groups					
Cyp46a1	Cytochrome P450, family 46, subfamily a, polypeptide 1	NM_001108723	1.2	1.6	−0.4
Lif	Leukemia inhibitory factor	NM_022196	1.1	1.3	−1.1
Itih4	Inter alpha-trypsin inhibitor, heavy chain 4	NM_019369	0.7	1.8	−0.5
RICS	RhoGAP involved in beta-catenin-N-cadherin and NMDA receptor signaling	XM_001056872	0.7	1.5	−0.4
Alpl	Alkaline phosphatase, tissue-nonspecific	NM_013059	0.6	1.5	−1.5
Lrrc4b	Leucine rich repeat containing 4B	XM_001077685	0.5	1.4	−1.0
Cmya1	Cardiomyopathy associated	XM_001077697	0.5	1.2	−0.4
Slc13a3	Solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3	NM_022866	0.5	1.2	−0.9
LOC683029	Hypothetical protein LOC683029	XM_001064175	0.5	1.3	−0.8
F2	Coagulation factor II	NM_022924	0.4	1.2	−0.5
Phf2	PHD finger protein 2	NM_001107342	−1.3	−2.5	0.5
Zfp68	Zinc finger protein 68	NM_001107128	−0.5	−2.5	2.7
RGD1309621	Similar to hypothetical protein FLJ10652	XM_001075984	−1.6	−2.1	4.1
Psip1	PC4 and SFRS1 interacting protein 1	NM_175765	−0.7	−2.1	1.1
Stx17	Syntaxin 17	NM_145723	−1.3	−2.1	0.4
Trim14	Tripartite motif protein 14	NM_001107934	−1.9	−2.1	0.9
RGD1561825	Similar to CDNA sequence BC020077	XM_001056277	−0.9	−2.0	1.2
Echdc1	Enoyl Coenzyme A hydratase domain containing 1	NM_001007734	−1.1	−1.9	1.1
Pbk	PDZ binding kinase	NM_001079937	−1.4	−1.8	0.8
Kcnip1	Kv channel-interacting protein 1	NM_022929	−0.4	−1.8	0.8

3.4. Proteomic analysis of the testis during early toxicity

Together with global gene expression changes in the testis after DNB treatment, we also found proteomic changes during testicular toxicity. Total proteins in testicular samples from two controls and three DNB-treated animals were separated by 2-DE. A comparison of the gels between the DNB-treated groups and the controls revealed 101 spots representing differentially expressed proteins with a greater than two-fold change in the DNB-treated groups (one-day group, 36 spots; three-day group, 31 spots; and seven-day group, 30 spots). In the present study, fewer differentially expressed spots were identified compared with other samples, such as those from the liver in a previous study of hepatotoxicity [21]. Additionally, many proteins were down-regulated in the DNB-treated groups. We focused on early testicular toxicity, and 36 protein spots, including four up-regulated and 32 down-regulated spots, were subjected to MALDI-TOF or MALDI-TOF/TOF following in-gel tryptic digestion. A representative 2-D gel and spot images are shown in Fig. 5; the identified proteins are listed in Table 4.

As shown in Table 5, four proteins, including ACAA2, LRRC23, ABHD10, and C5ORF9, were up-regulated, while 32 proteins, including WDR77, PIN1, FABP, TPI1, GAPDHS, and PPP1CA, were down-regulated in the DNB-treated groups after one day. Using 30 proteins of known function, protein interactions were analyzed using IPA based on its own network algorithm. As shown in Fig. 6A, biofunctions of differentially expressed proteins involved mainly carbohydrate metabolism, cell cycle and cell death, and cellular assembly and organization. Network analysis of these proteins showed that 12 proteins were first matched with carbohydrate metabolism and cellular development (Fig. 6B). Proteins such as ACAA2, ABHD10, KRP10, and ARPC4 interact directly or indirectly with each other and with core modulators such as PPP1CA. Second, eleven proteins were matched with cancer and reproductive system disease (Fig. 6C). For reproductive system disease, PIN1 may be important among these proteins. The network analysis for differentially expressed proteins suggested that proteins related to carbohydrate metabolism, cell death or proliferation respond first to early testicular toxicity.

4. Discussion

In recent years, the incidence of male infertility has increased due to the sudden rise in environmental pollutants, as well as genetic predisposition and the modern diet. Reproductive toxicants that impair reproductive function directly can damage testicular cells or disrupt the homeostasis of the endocrine system [31]. Several chemicals have a direct adverse effect on testicular cells:

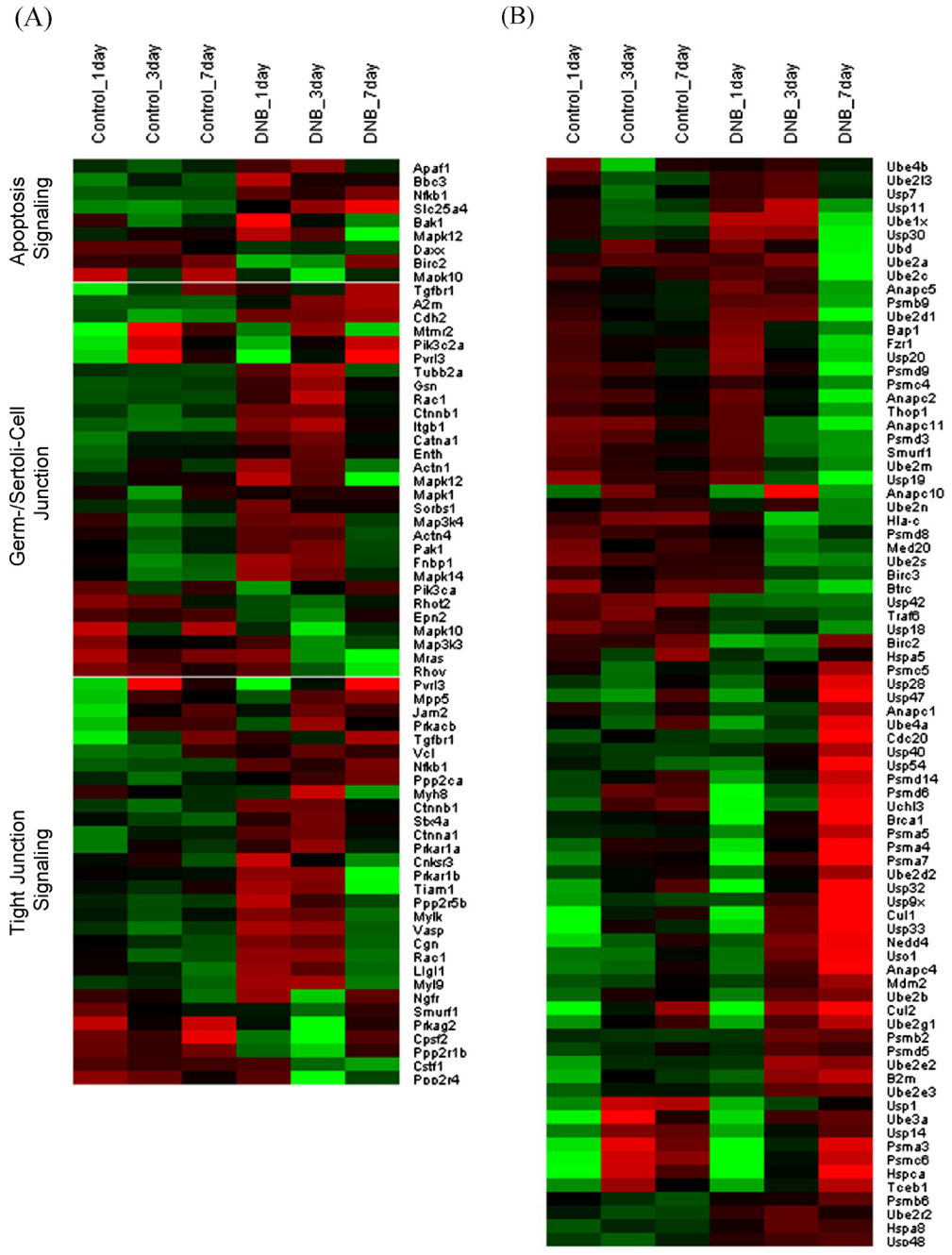

Fig. 4. Genes matched into germ cell/Sertoli cell junction signaling pathways in the three-day DNB-treated group. Red and green indicate up- and down-regulation in the DNB-treated groups, respectively. A mixed color indicates an inconsistent expression pattern (*e.g.*, isotype or family members of target genes).

ethane dimethane sulfonate is a Leydig cell-specific toxicant in rodent models, while several toxicants, including DNB and cadmium chloride, directly impair Sertoli cells, which play crucial roles in spermatogenesis by forming the blood-testis barrier and supporting germ cell maturation [3,7,29]. Histologic observations have shown that DNB causes testicular toxicity in rodent models by targeting Sertoli cells; however, the molecular mechanism remains unclear.

The integrative analysis of gene and protein expression changes was expected to give insight into the overall molecular mechanisms of testicular toxicity induced by DNB treatment. Well-defined phenotypes for progressive toxicity are needed to interpret the expression patterns obtained from microarray and proteomic experiments. In the present study, the decrease in organ weight and histopathologic findings indicated that testicular toxicity occurred time-dependently in the middle-dose group after DNB treatment. First, we analyzed our microarray data using a two-fold change and $P < 0.05$ as cutoff values. In total, 2866 genes differentially expressed in the one-, three-, or seven-day DNB-treated group were identified, but 1027 of the genes were not functionally annotated using IPA. We thus lowered the cutoff value to a >1.3-fold change ($P < 0.05$), but hierarchical clustering showed that the sample trees were well distinguished among each group and the overall gene expression patterns were similar under stringent cutoff conditions. Commonly regulated genes, such as *Testin*, *Tnfrsf12a* and *Nes*, which are usually up-regulated during toxicity involving intercellular junction or cell death [32–34], were also up-regulated in the DNB-treated groups. Interestingly, Wnt4 was significantly up-regulated in the testis. It has been reported that *Wnt4* plays a concerted role in both the control of female and male sexual development [35].

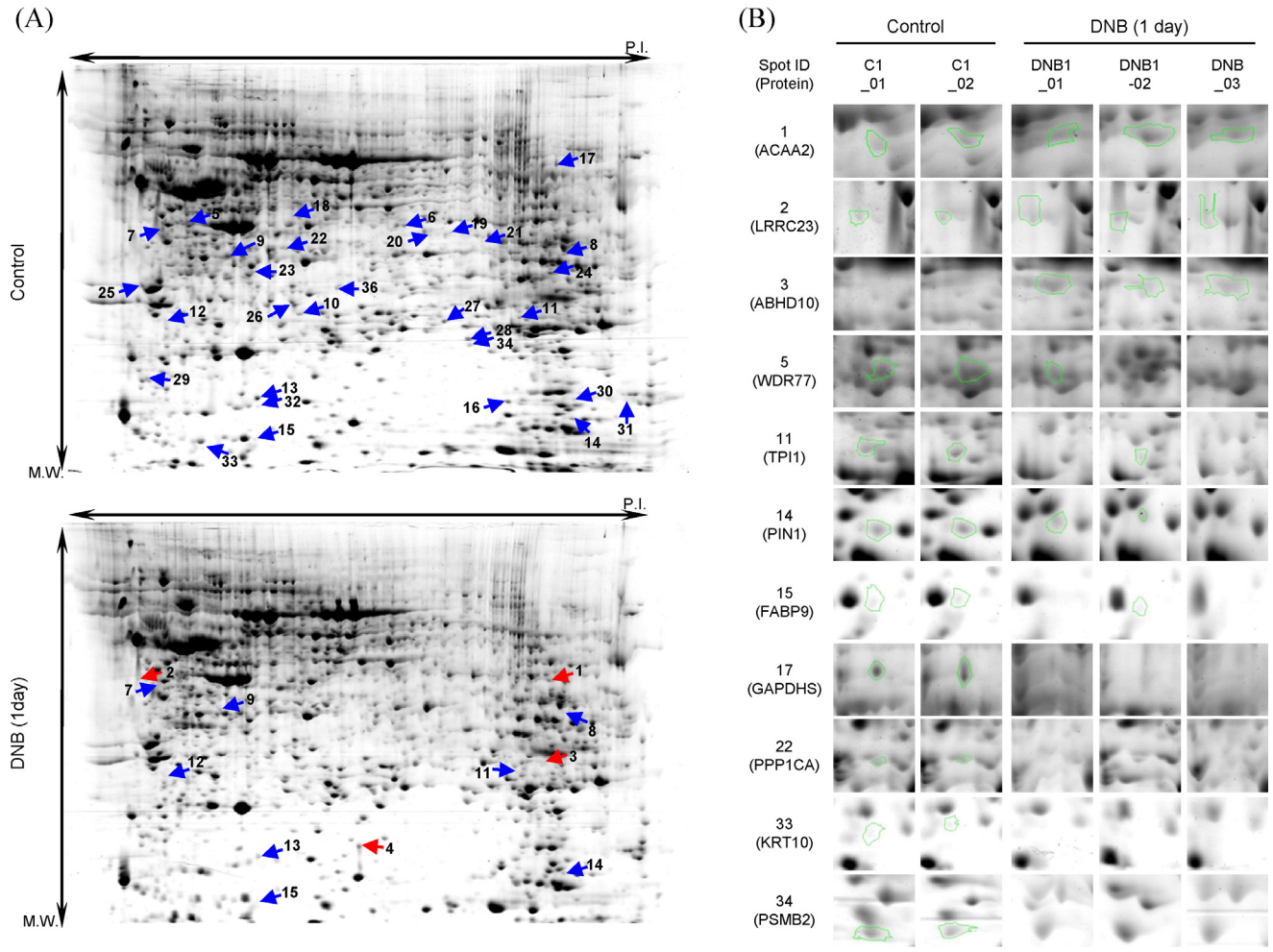

Fig. 5. Comparison of 2-D gel images in the one-day DNB-treated group with corresponding controls. (A) Overall maps of representative 2-D gels for two controls and three DNB-treated samples. (B) Representative spots of differentially expressed proteins in the one-day DNB-treated group.

Table 4
Major biofunctions of genes altered during testicular toxicity.

Molecular and cellular functions	*P*-value	No. of genes
1 day		
Cell death	2.44E-04–4.33E-02	202
Cellular development	4.19E-04–4.33E-02	135
Cell cycle	1.34E-03–4.33E-02	111
Gene expression	1.38E-03–4.33E-02	39
Cell-to-cell signaling and interaction	1.80E-03–4.33E-02	34
3 day		
Cell death	3.13E-04–4.98E-02	197
Amino acid metabolism	3.77E-04–4.98E-02	33
Post-translational modification	3.77E-04–4.71E-02	21
Small molecule biochemistry	3.77E-04–4.98E-02	102
Cell morphology	4.20E-04–4.71E-02	61
7 day		
DNA replication, recombination, and repair	1.88E-08–4.46E-02	235
RNA post-transcriptional modification	4.46E-05–3.35E-02	92
Cell cycle	7.07E-05–4.89E-02	372
Cellular movement	2.58E-04–4.89E-02	64
Cellular assembly and organization	1.48E-03–4.46E-02	198

The IPA computes *P*-values by comparing the number of molecules of interest relative to the total number of occurrences of these molecules in all functional annotations stored in the ingenuity pathways knowledge base (Fisher's exact test with the *P*-value adjusted using the Benjamini–Hochberg multiple testing correction).

The expression of another Wnt family member, *Wnt2*, which is associated with cellular development [36], was down-regulated in the seven-day treated group. Conversely, the expression of Inhibin B (*Inhbb*), a Sertoli cell-specific marker, did not change over the cutoff values, although *Inhbb* was down-regulated slightly in the seven-day group. Thus, *Inhbb* may not be a sensitive genomic biomarker for testicular toxicity. *Cyp46a1*, which is involved in cholesterol catabolism, was up-regulated during early toxicity but its expression was down-regulated at later phases. Other genes encoding metabolic enzymes such as those of the *Cyp2s/t/u* subfamily (*Cyp11a1*, *Cyp26a/b1*, and *Cyp27b1*) were also down-regulated in the seven-day treated group. Genes encoding P450 oxidoreductase (*Por*), several glutathione redox enzymes, including glutathione S-transferase α subunit 4 (*Gst4*), and glutathione reductase (*Gsr*), were up-regulated while the gene encoding glutathione synthetase was down-regulated in the seven-day treated group. Additionally, genes encoding thioredoxin 1 (*Txnl*) and sulfiredoxin 1 (*Srxn1*), which are usually overexpressed when toxicity occurs, were up-regulated at the early or late phase in the DNB-treated groups. The gene encoding S100 calcium binding protein A10 (calpactin, *S100a10*), which is involved in cell cycle progression, was up-regulated according to the degree of toxicity; interestingly, *S100a9* (calgranulin B) was up-regulated in the early phase, but its expression decreased in the late phase. Generally, genes associated with inflammatory or immune responses were highly regulated

Table 5
Identification of proteins altered during testicular toxicity after DNB exposure.

Spot ID	Symbol	Mascot MS Score*	Mascot MS/MS Score**	Acc. no.	Protein name
Up-regulated proteins in the DNB-treated group					
#1	ACAA2	128	38	gi|18426866	Acetyl-coenzyme A acyltransferase 2
#2	LRRC23	69		gi|61557152	Leucine rich repeat containing 23
#3	ABHD10	76		gi|56971308	RGD1308084 protein
#4	C5ORF49	65		gi|109460671	Chromosome 5 open reading frame 49
Down-regulated proteins in the DNB-treated group					
#5	WDR77	56		gi|32527757	WD repeat domain 77
#6	OBCAM	74		gi|149056121	Similar to opioid binding protein/cell adhesion molecule precursor
#7	FERMT1	63		gi|157817805	Fermitin family homolog 1
#8	C2	62		gi|149028002	Complement component 2, isoform CRA_a
#9	PPA1	66		gi|149038730	Pyrophosphatase (inorganic) 1
#10	–	75		gi|230919	Chain A, the structure of rat mast cell protease Ii At 1.
#11	TPI1	71	92	gi|38512111	Triosephosphate isomerase 1
#12	DUSP23	72		gi|34880952	Predicted: similar to dual specificity phosphatase 23
#13	PLCB4	66		gi|12643487	Phospholipase C, beta 4
#14	PIN1	75		gi|157817696	Peptidylprolyl cis/trans isomerase, NIMA-interacting 1
#15	FABP9	64	28	gi|12408304	Fatty acid binding protein 9, testis
#16	C9ORF9	54		gi|31745475	Chromosome 9 open reading frame 9
#17	GAPDHS	145	140	gi|13027414	Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating)
#18	LOC685817	74		gi|109512820	Predicted: similar to myosin-9B (Myosin IXb)
#19	C2ORF71	61		gi|109477957	Chromosome 2 open reading frame 71
#20	–	68		gi|149056180	rCG53732, isoform CRA_b
#21	FARS2	79		gi|149045198	Phenylalanine-tRNA synthetase 2
#22	PPP1CA	56		gi|4506003	Protein phosphatase 1, catalytic subunit, alpha isoform
#23	TPI1	64	133	gi|74271820	Triosephosphate isomerase
#24	CHCHD6	87		gi|149036711	Coiled-coil-helix-coiled-coil-helix domain containing 6
#25	LMO7	65		gi|46811858	LIM domain 7
#26	ZNF672	75		gi|56090447	Zinc finger protein 672
#27	LOC652956	56		gi|83320109	p55 protein
#28	CPS1	57		gi|8393186	Carbamoyl-phosphate synthetase 1, mitochondrial
#29	ANKRD2	62		gi|157820209	Ankyrin repeat domain 2
#30	ARPC4	81		gi|5031595	Actin related protein 2/3 complex, subunit 4, 20 kDa
#31	–	61		gi|149030589	rCG51817
#32	–	64		gi|149052643	rCG33456, isoform CRA_d
#33	KRT10	99	65	gi|57012436	Keratin 10
#34	PSMB2	104	149	gi|8394079	Proteasome (prosome, macropain) subunit, beta type 2
#35	PDXP	71		gi|85541051	Pyridoxal phosphate phosphatase
#36	NIT	172	61	gi|128485833	Nitrilase 1 isoform a

Proteins differentially expressed (>twofold) compared to controls are represented. Fold change was calculated from the quantification of relative % vol values of matched spots using the ImageMaster Platinum 6.0 image-analysis software. The selected protein spots were excised from Coomassie-blue-stained gels, digested with trypsin and then identified by MS and MS/MS analysis. Peptide mass fingerprinting and a MS/MS ion search was performed using the Mascot search engine. The Mascot score was considered significant if the probability of a random match was less than 5% ($P < 0.05$), as follows:

* Protein scores greater than 55 (automatically) or 61 (manually) are significant ($P < 0.05$).

** Individual ion scores above 28 indicate identity or extensive homology ($P < 0.05$).

during early toxicity, but gene expression profiling for testicular toxicity is slightly different from other toxicity phenomena such as hepatotoxicity and nephrotoxicity. That testicular toxicity occurs in distinct regions may be due to the blood-testis barrier. However, standard genetic markers of cell death and proliferation such as *Testin*, *Tnfrsf12a*, and *S100a10* were up-regulated in the DNB-treated groups. Oxidative stress may also have been triggered by DNB, based on changes in the expression of genes related to Nrf2-mediated oxidative stress.

Biofunction and canonical pathway analysis revealed that the expression of genes related to the cell cycle and cell death were altered during early testicular toxicity. Except for genes belonging to the canonical pathway of HIV1-induced apoptosis, other pro- or anti-apoptotic genes were also deregulated during early testicular toxicity. Pro-apoptotic death-associated protein kinase 1 (*Dapk1*) was up-regulated, whereas anti-apoptotic genes, including Bcl2-associated athanogene 3 (*Bag3*), Bcl2-like 1 (*Bcl2l1*), Bcl2-like 10 (*Bcl2l10*), baculoviral IAP repeat-containing 5 (*Birc5*), Bcl2/adenovirus E1B 19 kDa interacting protein 2 (*Bnip2*), and thioredoxin-related transmembrane protein 1 (*Tmx1*) were down-regulated during early testicular toxicity. Conversely, germ cell/Sertoli cell junction and tight junction signaling were identified with the highly ranked canonical pathway during early testicular toxicity. Germ cell/Sertoli cell junction signaling is important for complex biochemical events such as spermatogenesis. Many types of dynamic junctions exist in the testis, including those between Sertoli cells, Leydig cells, Sertoli/germ cells, and testicular cells/the extracellular matrix [37]. Germ cell/Sertoli cell junctions include cadherin-cadherin junctions, laminin-Itg-α6 and Itg-β1-nectin junctions. In the DNB-treated group, genes encoding components of the cadherin/Ctnn and integrin/FAK protein complex were up-regulated, while signaling molecules related to actin polymerization, cofilin disintegration and cell adhesion were sequentially regulated during early testicular toxicity. However, Fiorini et al. [29] reported that proteins between Sertoli cells that are associated with tight (occludin; *Ocln* and zonula occludins-1; *Tjp1*), anchoring (N-cadherin; *Cdh2*), and gap (connexin 43; *Gja1*) junctions were specific targets for testicular toxicants in the rat Sertoli SerW3 cell line. They also showed that DNB did not affect the levels of occludin (*Ocln*) and zona occludens 1 (*Tjp1*), while connexin 43 (*Gja1*) was decreased in DNB-treated Sertoli cells. In the present study, the transcriptional levels of genes encoding junction proteins was compared with those reported by Fiorini et al. [29]; *Gja1* was down-regulated, *Cdh2* was up-regulated and *Tjp1* was unaltered during early testicular toxicity. However, connexin 30 (*Gjb6*) was significantly down-regulated at all time points, and *Jam2* was up-regulated during early testicular toxicity. Fiorini et al. [29] reported that various reproductive toxicants did not consistently

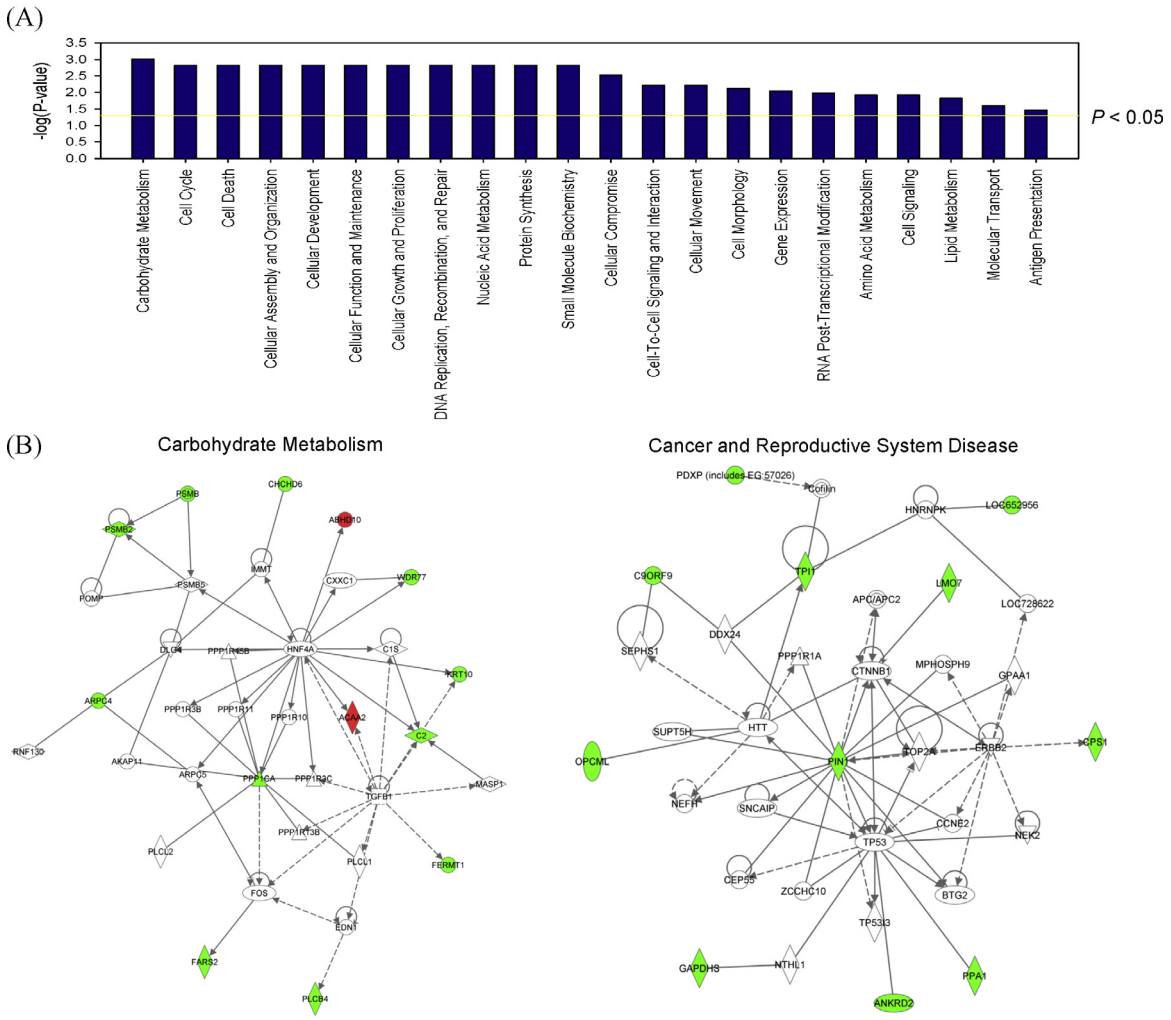

Fig. 6. Networks of proteins with altered expression profiles in the 1-day DNB-treated group. (A) Biofunction of 30 differentially expressed proteins in the 1-day DNB-treated group. (B) Twelve of the proteins with aberrant expression profiles are associated with carbohydrate metabolism and cellular development. (C) Eleven dysregulated proteins are associated with reproductive system disease. The red coloration represents up-regulated proteins and green indicates down-regulated proteins. (For interpretation of the references to color in figure legend, the reader is referred to the web version of the article.)

affect the level of tight junction proteins. The transcriptional level of genes encoding different tight junction proteins could provide information about alterations in Sertoli cell interactions induced by DNB. Canonical pathway analysis also demonstrated that many of the differentially expressed genes during late testicular toxicity (seven-day group) were involved in ubiquitination. The protein ubiquitination pathway is tightly regulated in the testis to ensure proper developmental programming and normal spermatogenesis. Tengowski et al. [30] also reported that ubiquitination-related genes were differentially expressed in DNB-exposed rats, which is consistent with our findings.

Proteomic analysis in the testis revealed that the total number of differentially expressed spots decreased time-dependently after DNB treatment. The expression of most identified spots was also down-regulated at all time points in the DNB-treated group. The number of differentially expressed spots and their expression patterns may vary depending on the severity of toxicity and treatment with various reproductive toxicants [23,38]. A comparison of the protein and gene expression changes indicated that *Acaa2* and *Wdr77* were consistently up- and down-regulated in the DNB-treated group, respectively; however, the transcriptional level of most proteins identified did not correlate statistically with protein expression. Several identified proteins were involved in spermatogenesis: GAPDH and TPI play roles in regulating glucose metabolism in both primary spermatocytes and spermatids [39,40], C9ORF9 (RSB-66) has been reported as a novel round spermatid-specific protein [41], and CPS1 has been reported to be highly expressed in human testis [42]. The down-regulation of these proteins during early testicular toxicity implies that these proteins are early markers of testicular toxicity. However, proteins related to apoptosis or cell death such as ACAA2, PIN1, FABP9, and PPP1CA were also identified in the DNB-treated groups [43–46]. ACAA2 is up-regulated in the testis during ethane dimethane sulfonate-induced early testicular toxicity, which targets Leydig cells (data not shown). Presumably, these proteins are initially regulated in the testis when cell death or apoptosis occurs. Additionally, we propose that PIN1 and PPP1CA are core regulators of this phenomenon based on our network analysis.

In summary, in this study we produced the first comprehensive gene and protein profile of testicular toxicity after DNB exposure in rats. We analyzed the biofunction, canonical pathway, and network interactions of genes or proteins found to be differentially expressed during testicular toxicity. Our data suggest that genes involved in apoptosis or tight junction, or Germ cell/Sertoli cells, are regulated at the transcriptional level and proteins involved in carbohydrate metabolism and reproductive disease interact with each other during early testicular toxicity. During toxicity progression, many genes associated with ubiquitination are downregulated

dramatically. This integrative interpretation will further our understanding of the mechanism underlying testicular toxicity after DNB exposure.

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