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Original Article

Busulfan administration replicated the characteristics of the epididymal initial segment observed in mice lacking testis-epididymis lumicrine signaling

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Abstract. The physiological functions of the mammalian epididymis are typically regulated by the testes. In addition to sex steroids secreted by testicular Leydig cells, which act on the epididymis in an endocrine manner, there is a non-sex-steroidal signaling pathway known as the lumicrine pathway. This lumicrine signaling pathway involves ligand proteins secreted from germ cells within the testicular seminiferous tubules traversing the male reproductive tract, which induce epithelial differentiation in the epididymis. These findings prompted an inquiry into whether treatments influencing testis physiology can disrupt epididymal function by interfering with testis-epididymis communication. Busulfan, an alkylating agent commonly used to deplete testicular germ cells in reproductive biology, has not been sufficiently explored because of its effects on the epididymis. This study investigated the effects of busulfan administration on the proximal epididymis using histological and transcriptomic analyses. Notably, busulfan, as opposed to the vehicle dimethyl sulfoxide (DMSO), altered the morphology of the initial segment of the epididymis, leading to a reduction in the cell height of the luminal epithelium. RNA sequencing identified 185 significantly downregulated genes in the proximal epididymis of busulfan-administered mice compared to DMSO-administered mice. Comparative transcriptome analyses revealed similarities between the epididymal transcriptome of busulfan-administered mice and lumicrine-deficient mice, such as efferent-duct-ligated *W/Wv* and *Nell2*$^{-/-}$ mice. However, this differed from that of bilaterally orchidectomized mice, in which both the endocrine and lumicrine signaling pathways were simultaneously ablated. Collectively, these results suggested that the harmful effects of busulfan on the proximal epididymis are secondary consequences of the ablation of testis-epididymis lumicrine signaling.
Key words: Busulfan, Epididymis, Gene expression, Initial segment, Lumicrine

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The epididymis, a highly coiled epithelial duct, is part of the sperm transport route. After production in the testis, morphologically complete, but functionally immature, testicular spermatozoa are transported through the efferent duct toward the epididymis, where they undergo further functional maturation necessary for their full fertilizing ability [1–4]. If spermatozoa are not properly matured by the epididymis, they will not be able to acquire the cellular functions necessary for fertilization, eventually resulting in a significant decrease in male reproductive ability. Functional differentiation of the epididymis is influenced by extra-epididymal or testicular factors, involving both endocrine and non-endocrine mechanisms as signaling systems between the testis and epididymis. In endocrine regulation, sex steroids from the Leydig cells reach the epididymis through the bloodstream and bind to epididymal cell receptors [5–10]. In non-endocrine regulation, proteins secreted by testicular germ cells are transported to the epididymis via the reproductive tract and bind to receptors in the luminal epithelium [11–13]. This type of secretion signaling is known as “lumicrine” signaling [14].

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Recent studies have revealed the molecular mechanisms underlying lumicrine signaling [2, 13, 15–19]. In the testes, germ cells located inside seminiferous tubules secrete lumicrine factor proteins, such as neural epidermal growth factor–like like 2 (NELL2) and NELL2-interacting cofactor for lumicrine signaling (NICOL), into the seminiferous lumen. These lumicrine factors move from the testis through the efferent duct to the epididymis via luminal flow. In the proximal or initial segment (IS) of the epididymis, the lumicrine receptor ROS1 tyrosine kinase is activated upon ligand binding, which triggers epithelial differentiation and induces the expression of genes necessary for epididymal function. The unveiled molecular mechanism of lumicrine signaling suggests that abnormalities in the IS of the epididymis can arise not only from disruptions in the luminal connection between the testis and epididymis or the inactivation of lumicrine factor genes in the testis, but also from the depletion of testicular germ cells. Indeed, in *Kit*-mutant *W/Wv* mice, an azoospermic mouse line in which spermatogonial cells cannot enter meiosis, lumicrine signaling is ablated, and the epididymal IS does not differentiate [13].

Butane-1,4-diyl dimethanesulfonate (busulfan) is a chemotherapeutic drug used in the treatment of specific cancer types, notably leukemia and other blood disorders. It falls within the class of alkylating agents, operating its effects by disrupting DNA in cancer cells and hindering their growth and division. In reproductive biology, busulfan is often used to create an azoospermic testicular environment conducive to the transplantation of exogenous spermatogonial cells by depleting endogenous germ cells [20–23]. Based on the molecular mechanism

of lumicrine signaling described above, there is a possibility that the busulfan-induced depletion of testicular germ cells may inhibit lumicrine-singaling-induced epididymal IS differentiation. However, the details of these interactions have not yet been explored. In the present study, the effects of busulfan on the proximal epididymis were investigated by analyzing the epididymal histology and transcriptome of busulfan-administered animals and comparing them with those of lumicrine- and endocrine-deficient animal models.

Materials and Methods

Animals

Male B6D2F1 and *W/Wv* mice were purchased from Japan SLC (Hamamatsu, Japan). *Nell2*-knockout mice were generated as previously described [13]. The animals were analyzed at the age of 14 weeks. Eight-week-old wild-type (WT) B6D2F1 males were administered dimethyl sulfoxide (DMSO) (Nacalai Tesque, Kyoto, Japan) or busulfan (Merck, Darmstadt, Germany) dissolved in DMSO (5 mg/ml) via intraperitoneal injection (40 mg busulfan/kg body weight, n = 3) and subjected to analyses 4 weeks after administration. Efferent duct ligation (EDL) was performed as described previously [24]. Briefly, the efferent ducts of 10-week-old B6D2F1 males were ligated unilaterally under anesthesia and the animals were analyzed 4 weeks after ligation. Bilateral orchidectomy or a sham operation was performed under anesthesia on 8-week-old WT B6D2F1 males, and the animals were subjected to analyses 4 weeks after the operation. For transcript and protein expression analyses, the IS was dissected together with the caput, and tissue dissection was performed as previously described [16,24]. This dissection method was used due to the difficulty in separately dissecting the IS from the caput epididymis, especially in mice in which IS differentiation is ablated. All experiments involving animals were approved by the Institutional Animal Care and Use Committee of Osaka University (approval number: Dou-Bi-R03-01-2) and were conducted in compliance with the university guidelines and regulations for animal experimentation.

Histology

Epididymal tissues were dissected, fixed with 4% formaldehyde (Electron Microscopy Sciences, Hatfield, England) in phosphate-buffered saline at 4°C overnight, dehydrated, and immersed in paraffin. The paraffin-embedded tissues were sectioned at 5 µm using a microtome (HM325; Microm, Walldorf, Germany). The sections were stained with hematoxylin and eosin and photographed using a system microscope (BX53; Olympus Tokyo, Japan). The height of the epithelial cells was measured (one measurement per epididymis) from the captured images.

Transcriptome analyses

Total RNA was isolated from the IS-caput of epididymides using an RNeasy Mini Kit (#74104; Qiagen, Hilden, Germany). On-column DNase treatment was performed during RNA purification using an RNase-free DNase set (#79254; Qiagen). The amount of RNA was determined by measuring the absorbance at 260 nm. RNA sequencing (RNA-seq) of epididymal transcripts was performed as follows. Libraries for sequencing were prepared from isolated RNA using a TruSeq stranded mRNA sample prep kit (#20020594; Illumina, San Diego, CA, USA) and sequenced on a NovaSeq6000 instrument (Illumina) using a 101 bp single-end mode. The obtained sequence reads were mapped to a mouse reference genome (mm10) using TopHat ver. 2.1.1 [25]. Cufflinks ver. 2.2.1 was used to calculate the fragments per kilobase of exon per million mapped reads values for each gene [26]. The RNA-seq data obtained were deposited in the NCBI Gene Expression Omnibus database under accession code GSE247820. The obtained IS-caput epididymal transcriptomes of DMSO- or busulfan-administered mice were compared with those of *W/Wv* and *Nell2*$^{-/-}$ mice and mice with EDL or bilateral orchidectomy, which are available from the NCBI Gene Expression Omnibus database under accession numbers GSE232898 and GSE247764. Plots and heat map representations of gene expression levels were generated using Microsoft Excel 2019 (Microsoft Corporation, Redmond, WA, USA).

Antibodies

The following commercially available antibodies were used: rabbit polyclonal anti-a disintegrin and metalloproteinase domain-containing protein 28 (ADAM28) (#22234-1-AP; Proteintech, Rosemont, IL, USA), mouse monoclonal anti-glutathione peroxidase 5 (GPX5) (sc-376877; Santa Cruz Biotechnology, Dallas, TX, USA), mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (#s sc-32233, Santa Cruz Biotechnology), and rabbit polyclonal anti-ribonuclease 10 (RNASE10) (#PA5-62646; Thermo Fisher Scientific, Waltham, MA, USA) as primary antibodies, and peroxidase-conjugated goat polyclonal anti-rabbit IgG (#111-036-045, Jackson Immunoresearch, West Grove, PA, USA) and goat polyclonal anti-mouse IgG (#115-036-062, Jackson Immunoresearch) as secondary antibodies. A rabbit polyclonal antibody against ovochymase 2 (OVCH2) was obtained as previously described [13]. An anti-sperm-associated antigen 11 B (SPAG11B) antibody was raised in rabbits by immunization with the synthetic peptide, Ac-KDEFPARGVNGSQLLHHRVKRC-NH_2, which corresponds to amino acid residues 40–60 of mouse SPAG11B, followed by an additional Cys residue conjugated with keyhole limpet hemocyanin. The raised antibody was affinity purified using SulfoLink Coupling Resin (#20401, Thermo Fisher Scientific), to which the antigen peptide was covalently conjugated. The dilution conditions for the antibodies are summarized in Supplementary Table 1.

Protein expression analyses

Dissected mouse tissues were homogenized in lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, and 1% Triton X-100) containing a protease inhibitor cocktail (#25955-24; Nacalai Tesque) and a phosphatase inhibitor cocktail (#07575-51, Nacalai Tesque). The homogenates were centrifuged at 12,000 × *g* at 4°C for 15 min, and the resulting supernatants were recovered as crude tissue protein extracts. The protein concentrations of the crude tissue protein extracts were determined using a Pierce BCA protein assay kit (#23227, Thermo Fisher Scientific). Twenty micrograms of the extracted proteins was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions using an e-PAGEL precast gel (#E-T/R/D520L; Atto, Tokyo, Japan). Precision Plus Protein Dual Color Standards (#1610374; Bio-Rad, Hercules, CA, USA) were used as molecular weight standards. The separated proteins were electrotransferred onto polyvinylidene difluoride membranes using the Trans-Blot Turbo transfer system (#1704150J1, Bio-Rad) and a Trans-Blot Turbo Mini apparatus (#1704156, Bio-Rad). The membranes were then blocked with 3% bovine serum albumin (#01859-47, Nacalai Tesque) and 0.05% (w/v) Tween20 (#35624-02, Nacalai Tesque) in Tris-buffered saline at room temperature for 30 min and incubated with primary antibodies at the indicated dilutions at 4°C overnight. The bound antibodies were detected by incubation with peroxidase-conjugated secondary antibodies and chemiluminescence using Chemi-Lumi One Super (#02230, Nacalai

Tesque). Chemiluminescent signals were detected and imaged using an Amersham ImageQuant 800 system (Cytiva, Tokyo, Japan).

Statistical analyses

Epithelial cell height was evaluated using a one-way analysis of variance and two-tailed Student's *t*-tests under the assumption of unequal variance using Microsoft Excel 2019. The transcript levels of each gene were evaluated using two-tailed Student's *t*-tests under the assumption of unequal variance using Microsoft Excel 2019.

Results

Busulfan administration altered the histology of the IS of the mouse epididymis

Busulfan (40 mg/kg body weight) dissolved in DMSO or DMSO alone was intraperitoneally administered once to 8-week-old mice, and the IS-caput of the epididymides were isolated after 4 weeks. The isolated tissues were embedded in paraffin and sectioned for histological analyses. A tall luminal epithelium was prominent in the IS of the WT mouse epididymis (Fig. 1A). The height of the IS luminal epithelium was unaffected in the DMSO-treated mice (Fig. 1B). In contrast, the IS luminal epithelial height was reduced by busulfan administration (Fig. 1C). These observations indicated that busulfan affects the histology of the IS of the epididymis.

A reduced cell height of the IS luminal epithelium has been observed in various experimental animal models in which the action of the testis on the epididymis is inhibited. In *W/Wv* and *Nell2*$^{-/-}$ mice and mice with EDL, where testis-epididymis lumicrine signaling is experimentally or genetically impaired, the IS luminal epithelium cell height was reduced (Figs. 1D–F), as previously observed [13, 16]. Similarly, in bilaterally orchidectomized mice, where both testis-derived lumicrine and endocrine signaling are impaired, the IS luminal epithelium cell height was reduced (Fig. 1G). Collectively, these observations suggested that the histological abnormalities caused by busulfan administration may arise as a consequence of lumicrine and/or endocrine singaling deficiency.

Busulfan administration altered the IS-caput epididymal transcriptome

The effect of busulfan administration on epididymal gene expression was investigated using transcriptome analysis. The IS-caput of the epididymides were dissected from WT, DMSO-administered, and busulfan-administered mice and total tissue RNA was subjected to RNA-seq analyses. The results are summarized in Supplementary

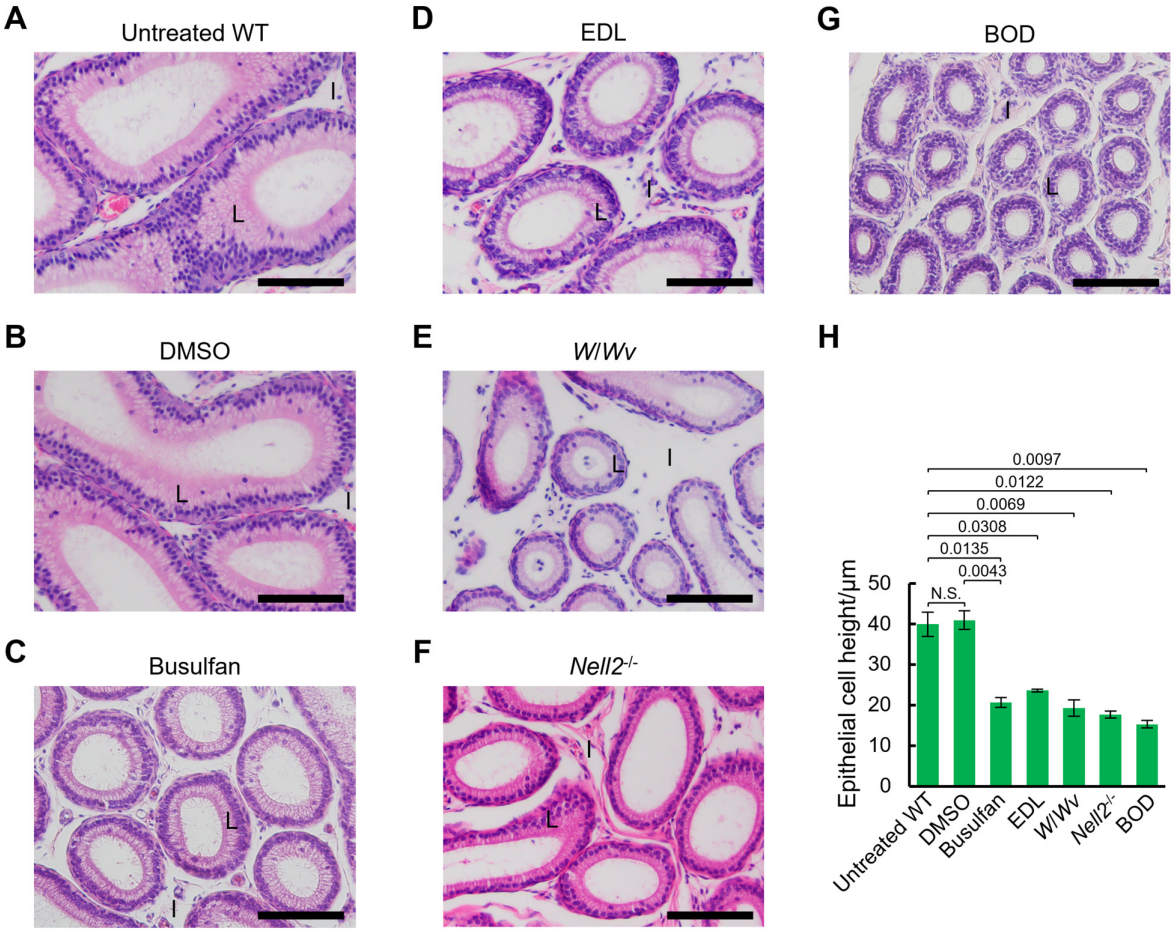

Fig. 1. Histology of the initial segment of the epididymis of busulfan-treated mice. A–G, Hematoxylin and eosin (HE)-stained sections of IS-caput of the epididymides from wild-type (WT) (A), dimethylsulfoxide (DMSO)-treated (B), and busulfan-treated (C) mice with efferent duct ligation (EDL) (D), *W/Wv* mice (E), *Nell2*$^{-/-}$ mice (F), and bilaterally orchidectomized mice (G). L, luminal epithelium; I, interstitial tissue. Bars, 100 μm. (H) Average cell height of the initial segment (IS) of the luminal epithelium. All values are shown as the mean±standard error of the mean (n = 3). The results of one-way analysis of variance were: F(6, 14) = [36.797], *P* = 8.67E-08. The *P* values of the two-tailed Student's *t*-test are also shown. BOD, bilateral orchidectomy. N.S., not significant.

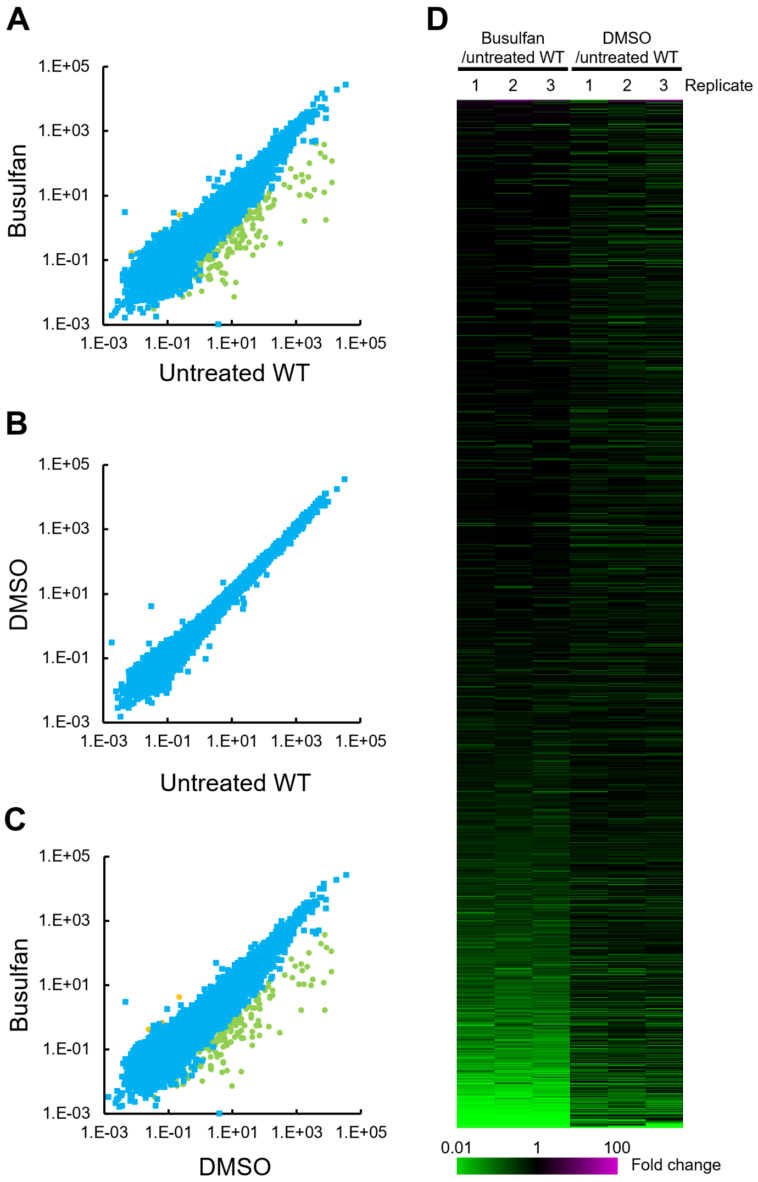

Fig. 2. RNA sequencing analyses of the IS-caput of the epididymis of DMSO- or busulfan-treated mice. A–C, RNA sequencing of the IS-caput of the epididymis from untreated WT *vs*. DMSO-treated (A), untreated WT *vs*. busulfan-treated (B), and DMSO-treated *vs*. busulfan-treated (C) mice. Fragments per kilobase of exon per million mapped reads (FPKM) values are plotted. Statistically significantly downregulated (fold change < 0.1, and Student's *t*-test P < 0.05) and upregulated (fold change > 10, and Student's *t*-test P < 0.05) genes are represented in green and yellow, respectively. (D) The fold change in gene expression levels in DMSO-treated *vs*. untreated WT mice (n = 3) and busulfan-treated *vs*. untreated WT mice (n = 3). Green and magenta represent downregulation and upregulation, respectively.

Data File 1. A comparison of IS-caput epididymal gene expression between WT and busulfan-administered mice revealed the significant downregulation of many genes (Fig. 2A). In busulfan-treated mice, 235 genes exhibited a significant reduction in expression levels of < 1/10 (Table 1). In contrast, no prominent differences were observed between WT and DMSO-administered mice (Fig. 2B). Only six genes were significantly reduced to less than 1/10 in DMSO-administered mice (Table 1), suggesting that the amount of DMSO used had a subtle effect on IS-caput gene expression, making DMSO-administered animals a suitable experimental negative control (Fig. 2C). A heatmap representation of the fold changes in gene expression in IS-caput of the epididymides from DMSO-administered and busulfan-administered mice (n = 3) visually highlighted the reproducible downregulation of many genes by busulfan injection (Fig. 2D). These results underscore the critical impact of busulfan injection on IS-caput epididymal gene expression. In subsequent transcriptome analyses, DMSO-treated animals served as negative controls for busulfan-treated animals.

Gene expression patterns in the IS-caput of the epididymis following busulfan administration resembled lumicrine-deficient phenotypes

There are two mechanisms that regulate IS epididymal cell differentiation and gene expression: an endocrine mechanism involving testis-derived sex steroids and a lumicrine mechanism involving secretory proteins transported through the lumen of the reproductive tract from the testis to the epididymis. Hence, the IS-caput epididymal transcriptome of busulfan-administered mice was characterized by comparisons with those of *W/Wv*, *Nell2*$^{-/-}$, and bilaterally orchidectomized mice and mice with EDL (Fig. 3). Gene downregulation in the IS-caput of the epididymis from busulfan-administered mouse resembled that of *W/Wv* and *Nell2*$^{-/-}$ mice and mice with EDL, suggesting a common mechanism of gene downregulation among these experimental groups. The expression levels of genes such as *Defb41*, *Eppin*, *Gm1110*, *Gpx5*, *Lcn12*, *Lypd8*, *Rhcg*, and *Teddm1b* [27–34], which were abundant in the DMSO-administered control IS-caput, were not critically affected by busulfan or other treatments interfering with lumicrine signaling, but were significantly downregulated by bilateral orchidectomy (see also Fig. 3). Thus, not all genes

Table 1. Summary of downregulated and upregulated genes in the IS-caput of the epididymis of mice after DMSO or busulfan administration

Treatment	Number of genes		
	Student's *t*-test P < 0.05	Downregulated genes Fold change < 0.1 Student's *t*-test P < 0.05	Upregulated genes Fold change > 10 Student's *t*-test P < 0.05
DMSO *vs*. Untreated WT	6,335	6	14
Busulfan *vs*. Untreated WT	3,537	235	10
Busulfan *vs*. DMSO	5,828	185	16

DMSO, dimethylsulfoxide; IS, initial segment; WT, wild-type.

Fig. 3. Comparative representation of genes downregulated in IS-caput of epididymides by busulfan treatment and other experimental treatments. Fold change in gene expression levels in the IS-caput of the epididymis compared between busulfan-injected mice, mice with EDL, *W/Wv* mice, *Nell2*$^{-/-}$ mice, and bilaterally orchidectomized mice. Green and magenta represent downregulation and upregulation, respectively. BOD, bilateral orchidectomy.

downregulated by bilateral orchidectomy were similarly affected by busulfan administration, indicating that endocrine regulation of epididymal gene expression by sex steroids was not significantly affected in busulfan-administered mice. In contrast, the number of genes upregulated by busulfan administration was very small compared to the number that was downregulated (Table 1), and the expression levels were still considerably low (Figs. 2A and C), indicating that busulfan administration had subtle effects on gene upregulation in the IS-caput of the epididymis.

Altered gene expression levels were analyzed by immunoblotting analysis of IS-caput proteins. Secreted proteins ADAM28, OVCH2, and RNASE10 are expressed in the epididymal IS in a lumicrine-signaling-dependent manner [13, 35]. The expression levels of these secreted proteins were also significantly diminished in the IS-caput of the epididymis of busulfan-administered mice, as observed in lumicrine-signaling-deficient *W/Wv* and *Nell2*$^{-/-}$ mice and mice with EDL, and lumicrine- and endocrine-signaling-deficient bilaterally orchidectomized mice (Fig. 4). The expression of GPX5 and SPAG11B, secreted proteins abundant in the epididymal IS, was regulated in a sex-steroid, endocrine-dependent, but lumicrine-signaling independent manner (Fig. 4) [30]. The expression levels of these secreted proteins in the IS-caput were critically affected in bilaterally orchidectomized mice, but not in busulfan-administered or lumicrine-signaling-deficient *W/Wv* and *Nell2*$^{-/-}$ mice and mice with EDL (Fig. 4).

Collectively, these results indicated that the IS-caput epididymal gene expression patterns of busulfan-treated animals were similar to those of *W/Wv* and *Nell2*$^{-/-}$ mice and mice with EDL, but unlike those of bilaterally orchidectomized mice, at both the transcript and protein levels.

Discussion

It has been well established that a single dose of busulfan impedes the propagation of germ cells in the testes. Previous studies have reported the toxic effects of busulfan on the epididymis, revealing that a single intraperitoneal administration of busulfan in mice reduces epididymal epithelial thickness and alters the expression levels of several genes related to epithelial integrity [36]. Notably, the morphology and transcriptome of the IS have not been thoroughly examined in these studies. In the present study, the effect of busulfan administration on the IS of the epididymis was examined, with a focus on its relationship with lumicrine signaling.

Previous studies have shown that many genes are expressed in the epididymis and are regulated by testicular endocrine and/or lumicrine signaling [6, 13, 16, 24, 31, 34, 37–210], providing an opportunity to perform comparative gene expression analyses. Notably, the IS of the epididymis in busulfan-administered mice

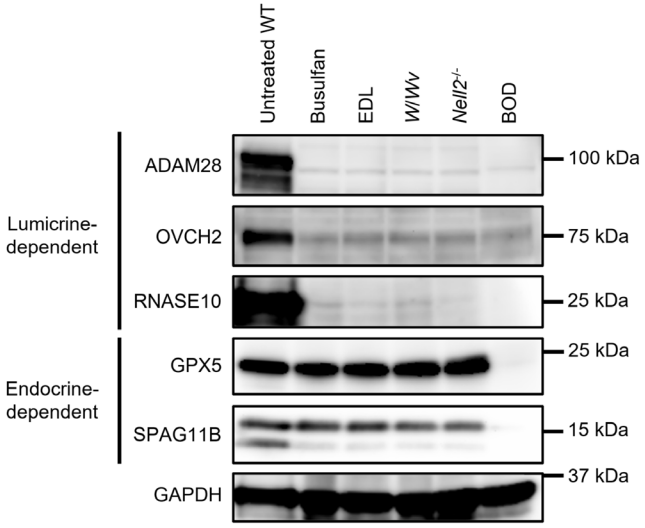

Fig. 4. Protein expression levels in the IS-caput of the epididymis of busulfan-treated and other experimentally treated mice. Immunoblot analyses of lumicrine-signaling-associated proteins ADAM28, OVCH2, and RNASE10 and endocrine-signaling-associated proteins GPX5 and SPAG11B in IS-caput epididymal lysates from untreated WT mice, busulfan-treated mice, mice with EDL, *W/Wv* mice, and *Nell2*$^{-/-}$ mice, and bilaterally orchidectomized (BOD) mouse. GAPDH immunodetection is also shown as an internal control.

closely resembled lumicrine-deficient states in terms of both histology and gene expression patterns. However, it differed distinctly from the IS of the epididymis in bilaterally orchidectomized mice, in which both endocrine and lumicrine signaling are simultaneously abolished. These findings suggested that busulfan administration did not critically affect testicular sex steroidal endocrine activity in the IS of the epididymis. Detailed transcriptome analyses of bilaterally orchidectomized mouse epididymides will be performed in future studies.

Given that busulfan depletes testicular germ cells, any indirect effect on the IS of the epididymis likely stems from germ cell depletion in the testes. This situation parallels the lumicrine signaling ablation observed in germ-cell-deficient *W/Wv* mice, testicular-lumicrine-factor-deficient *Nell2*$^{-/}$ mice, and mice with EDL in which luminal flow is impaired. These observations strongly suggest that the effects of busulfan on the epididymis, if secondary to testicular abnormalities, are due to lumicrine signaling insufficiency. It remains unclear whether the action of busulfan on the proximal epididymis is direct, indirect, or complex, as schematically summarized in Fig. 5.

An intriguing avenue for future research is to explore alterations in the IS of the epididymis when spermatogonial cells are transplanted into testes depleted of germ cells following busulfan administration. If such transplantation restores the IS of the epididymis to a normal state, it may be inferred that the effects of busulfan on the IS result from abnormal lumicrine signaling due to germ cell depletion in the testes.

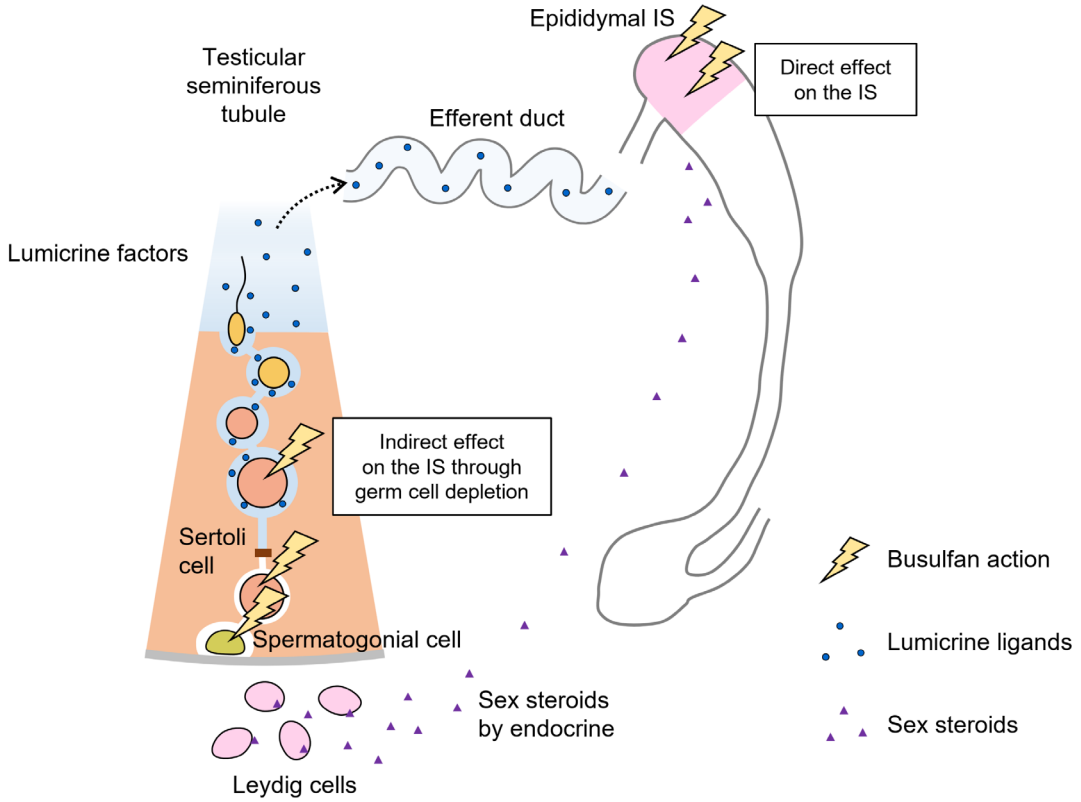

Fig. 5. A scheme representing the possible mechanism of action of busulfan on the IS of the epididymis. Lumicrine signaling is interfered with indirectly by busulfan administration, as a secondary consequence of testicular germ cell ablation. The endocrine action by Leydig cells appears to be unaffected by busulfan administration. The direct action of busulfan on the IS of the epididymis is also possible, although it is currently uncertain whether such an action causes IS defects.

The administration of busulfan has emerged as a practical experimental approach to disrupt testis-epididymis lumicrine signaling. As demonstrated in this study, the histology and gene expression pattern in the IS of the epididymis of busulfan-treated mice closely resembled those of lumicrine-signaling-ablated animals. While other experimental strategies exist to interfere with testis-epididymis lumicrine signaling, accessibility to mutant animals, such as *W/Wv*, *Nell2*$^{-/-}$, *Nicol*$^{-/-}$, and *Ros1*$^{-/-}$ mice, may pose challenges for some researchers owing to limited bioavailability. EDL requires surgical skills and should be performed under appropriate anesthesia, making it less universally applicable. In contrast, busulfan treatment is more convenient, requiring only a single intraperitoneal injection, without surgery or anesthesia.

In conclusion, our study explored the effect of busulfan on the epididymis and revealed similarities to lumicrine-deficient states. Likely acting through germ cell depletion, the effects of busulfan mimic the lumicrine signaling disruptions observed in other models. A more in-depth understanding of the effect of busulfan on the IS of the epididymis may present it as an alternative method for investigating testis-epididymis communication.

Conflicts of interests: The author declares no competing interests.

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