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Susceptibility of the Male Reproductive Tract Tissues of *Spag11a* Knockout Mice to DEN-Induced Tumorigenesis: A Histological Analysis

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Abstract

The Sperm Associated Antigen 11a (*Spag11a*), a caput epididymis-specific gene that codes for a secreted protein SPAG11A, is important for sperm maturation and epididymal immune responses. Using siRNA- and active immunisation-mediated ablation of its mRNA and protein and knockout models, we previously demonstrated its role in the proliferation of epididymal epithelial cells and the susceptibility of the epididymis to chemically induced tumorigenesis. However, the effect of its knockout on the susceptibility of other male reproductive organs to chemically induced tumorigenesis has not been reported. In this study, using *Spag11a* knockout mice, we report that the cauda epididymis, testis, prostate, and seminal vesicles of Spag11a knockout mice are more susceptible to DEN-induced carcinogenesis compared to their counterparts obtained from wild-type mice. Hyperplasia, anaplasia, dysplasia, neoplasia, and inflammation in the cauda epididymis, testis, prostate, and seminal vesicles were observed in *Spag11a* knockout mice, while these tissues of wild-type mice displayed normal anatomical structure. Among the tissues analysed, the prostate seems to be highly affected. Results of this study demonstrate that SPAG11A can influence the susceptibility of other male reproductive organs to tumorigenesis.

Keywords: DEN-Induced Tumorigenesis, Histological Analysis, *Spag11a* gene.

1. Introduction

The epididymal milieu comprises a variety of factors, including proteins that confer the motility and fertilising ability, resulting in sperm maturation. A wide variety of proteins have been reported to be secreted by the epididymis, which bind to the sperm surface to induce functionality of the male germ cells[1,2]. Further, the differential expression of genes and proteins along the length of the epididymis is reported[3,4]. Among the protein families identified, the Sperm associated antigen 11 (*SPAG11*) protein family emerged as one of the crucial factors for sperm maturation and function. In humans, the *SPAG11* gene (initially identified as human epididymal protein 2 (HE2)) is present on chromosome 8p23[5,6]. This gene emerged as a result of the fusion of two ancestral β-defensin genes and displays extensive alternate splicing to produce 20 isoforms[6]. Proteins of this family are implicated in various functions that include male germ cell maturation and innate immunity of the epididymis. Though there is a single *SPAG11A* gene in humans, in rodents, independent genes were identified, namely, *Spag11t*, *Spag11c* and *Spag11a* (*Bin1b or Spag11e*), and they are found to be localised on the sperm surface[7]. The mouse *Spag11a* gene product is involved in male germ cell maturation and function[8]. It is quite interesting to note that some of the SPAG11 proteins contain a conserved six cysteine motif, similar to that of β-defensins and exhibit potent antimicrobial activity, thereby contributing to the first line of defence in the epididymis[7,9].

The initial line of evidence that the *Spag11a* gene may play a role in the regulation of epididymal epithelial cell proliferation was demonstrated in our previous study. It was observed that epididymal epithelial cells in which *Spag11a* mRNA levels were suppressed by siRNA, exhibited increased proliferation; and conversely, immortalised caput cell lines in which *Spag11a* was overexpressed exhibited decreased proliferation[10]. Using

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active immunisation, we further demonstrated that suppression of SPAG11A protein rendered the epididymis more susceptible to chemically induced tumorigenesis in epithelial cells, promoting proliferation[10]. To provide further evidence, Spag11a knockout mice were generated, and the susceptibility of the epididymis to chemically induced tumorigenesis was tested. We observed that the caput epididymis of *Spag11a* knockout mice displayed histopathological changes that indicated initiation of tumorigenesis[11]. Since the gene product of the *Spag11a* gene, *i.e.*, SPAG11A, is a secreted protein, it is possible that it can have a role in the functioning of other organs of the male reproductive system. To test this hypothesis, we analysed the histopathological changes in the cauda, testis, prostate, and seminal vesicles of *Spag11a* knockout mice subjected to chemically induced tumorigenesis. We observed that the susceptibility of these tissues obtained from *Spag11a* knockout mice was higher than that of their wild-type counterparts.

2. Methodology

2.1 *Spag11a* Knockout Mice Generation

Heterozygous *Spag11a* knockout mice (C57BL/6NJ) were obtained commercially from the National Centre for Biological Sciences (NCBS), Bengaluru, India. The strategy adopted to generate these mice is described in our previous study[11]. Homozygous knockout mice in which exon 1 was deleted were obtained by backcrossing the heterozygous mice. The project #AJ-1/2015 for generating the heterozygous and homozygous mice was approved by NCBS-IAEC (R1-E), and the University of Hyderabad's Institutional Ethics Committee accepted the use of animals in the experimental study (UH/IAEC/SY/2021-22/09), respectively. The deletion of exon 1 of the *Spag11a* gene was confirmed by genotyping, and the lack of mRNA expression was confirmed by PCR and real-time PCR as described in our previous study[11].

2.2 Carcinogen Treatment

To determine the susceptibility of the male reproductive organs of wild type and *Spag11a* heterozygous and knockout mice (n = 3 in each group) to a low dose of carcinogen, diethylnitrosamine (DEN), was administered at a dose of 0.05 ppm in drinking water *ad libitum* for 12 weeks. The dose and duration were determined as per the protocol described earlier[12]. Mice without any carcinogen treatment (control; n = 3) were also maintained. At the end of the treatment period, mice were sacrificed, and the tissues (cauda epididymis, testes, seminal vesicles, and prostate) were collected for further analyses.

2.3 Histopathological Analyses

Hematoxylin and eosin staining was performed to evaluate the histopathological changes. Paraformaldehyde (4%) soaking for 24 h was used to fix the tissues, followed by washing in 70% ethanol and subsequent progressive dehydration in graded ethanol (80, 90, and 100%). Tissues were then encased in paraffin, and sections (five-micron) were generated on a microtome. Following an overnight incubation in isopropanol at 60°C, they were deparaffinised with xylene and progressively rehydrated with decreasing ethanol concentrations (100, 90, 80, 70, and 50%). After thorough washing with distilled water, they were treated with 1% hydrochloric acid for 30 s to allow differentiation. Staining was carried out with 0.2% Harris hematoxylin solution for 10 min, followed by counterstaining with 0.2% eosin Y solution for one minute and serially dehydrated with 50, 70, 80, 90, and 100% alcohol. After three xylene washes, the sections were mounted using xylene-based mounting media. Using a trinocular microscope, the sections were photographed. The histopathological changes were assessed in at least 25 areas for each section by a board-certified histopathologist unaware of the sample identity and experimental setup.

3. Results

3.1 Effect of *Spag11a* Knockout on Chemically Induced Carcinogenesis

Moderate proliferation of connective tissue, anaplastic nature and hyperplasia were evident in the cauda of *Spag11a* knockout mice, while normal morphology was observed in the heterozygous mice (Figure 1). In the testes of *Spag11a* heterozygous and knockout mice, considerable spermatogonial cell degeneration and infiltration of multinucleated cells were evident (Figure 2). The mucosal glands of the seminal vesicles of *Spag11a* heterozygous and knockout mice showed mild fibrosis and connective tissue growth (Figure 3). Among the tissues analysed, severe effects were observed in the prostate. In the prostate of *Spag11a* heterozygous mice, early-stage prostate cancer was characterised by dysplastic and anaplastic changes in basal and mucosal

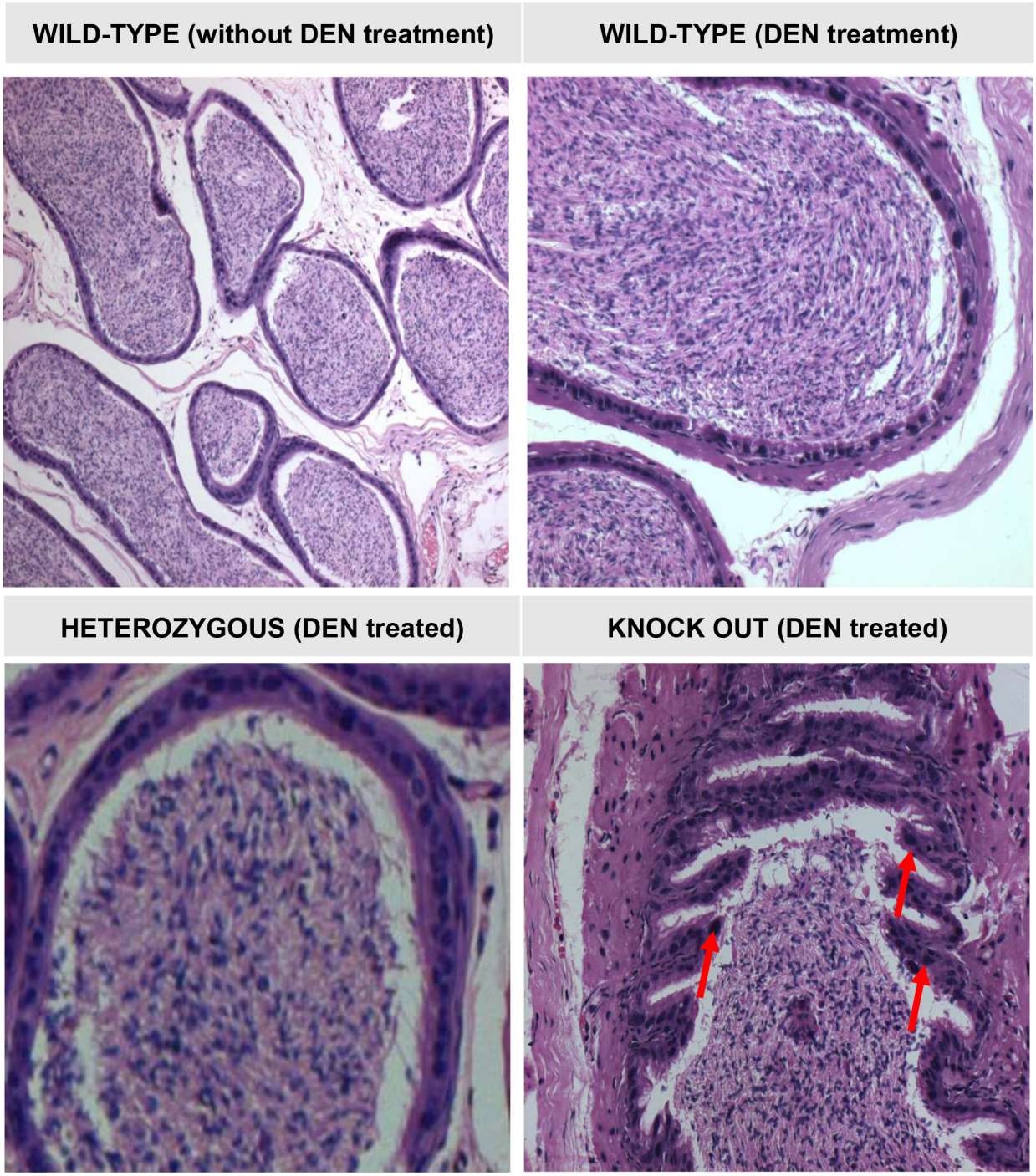


Figure 1. Histopathological changes in the cauda epididymis of DEN-treated wild-type, heterozygous, and knockout mice. Cauda epididymis from mice treated with DEN were fixed, and 5-micron sections were taken and stained with Hematoxylin and Eosin. Photomicrographs were taken in a phase contrast microscope. Arrows indicate the site of histopathological changes observed. Scale bar: 200 µm.

cells of prostate epithelium (Figure 4). Further, a basal cell tumour of the prostate with signs of aggressive neoplastic basal cells invading the entire mucosal folds and muscular region was observed. Dysplastic and anaplastic/basophilic changes in basal and mucosal cells of prostate epithelium and invasion of the submucosal layer, indicative of early-stage prostate cancer, were evident. In the *Spag11a* knockout mice, DEN treatment

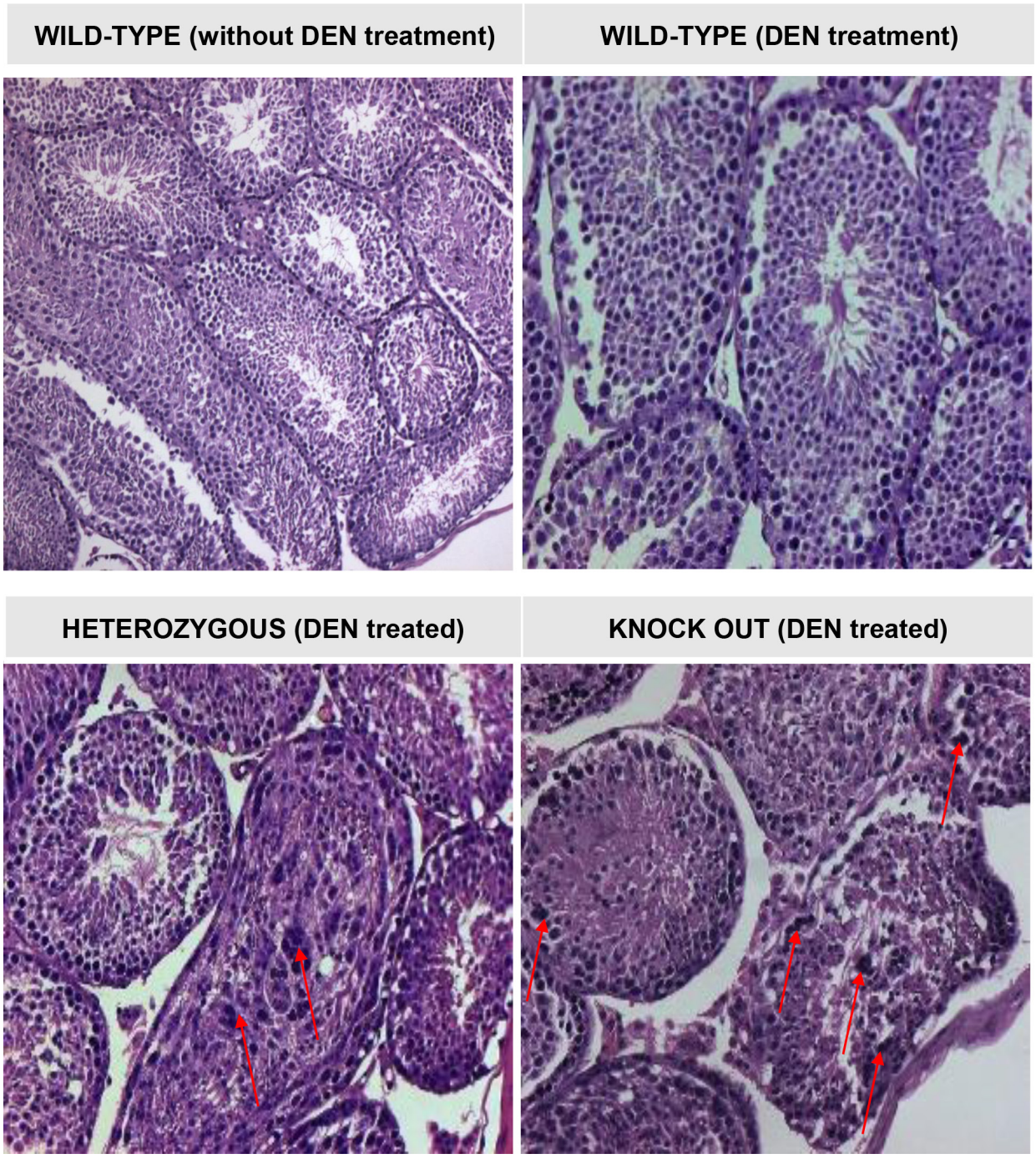


Figure 2. Histopathological changes in the testes of DEN-treated wild-type, heterozygous, and knockout mice. Testes from mice treated with DEN were fixed, and 5-micron sections were taken and stained with Hematoxylin and Eosin. Photomicrographs were taken in a phase contrast microscope. Arrows indicate the site of histopathological changes observed. Scale bar: 200 µm.

resulted in prostate tumours (Figure 4). Atypical basal cells of the prostate formed an intraluminal neoplasia in mucosal folds. The neoplastic cells were pleomorphic, basophilic, and had cytoplasm and nucleus ratio variations.

Neoplastic basal cells of prostate invading into the adjacent muscular layer, and a mild inflammatory reaction in the mucosal epithelial layer, with infiltration of plasma cells and a few anaplastic/basophilic epithelial cells were noticed. Spread of dysplastic epithelial cells observed

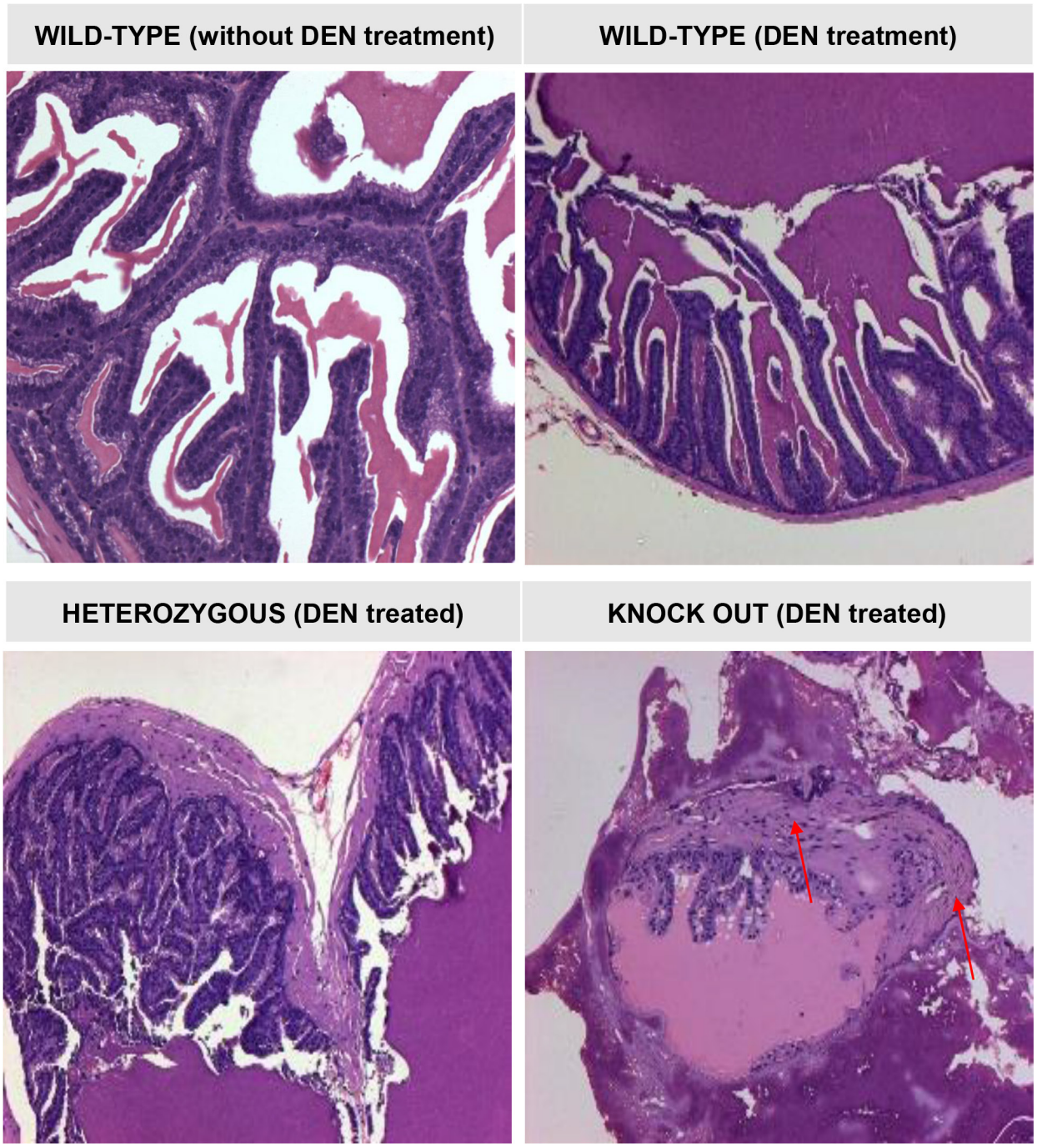


Figure 3. Histopathological changes in the seminal vesicles of DEN-treated wild-type, heterozygous, and knockout mice. Seminal vesicles from mice treated with DEN were fixed, and 5-micron sections were taken and stained with Hematoxylin and Eosin. Photomicrographs were taken in a phase contrast microscope. Arrows indicate the site of histopathological changes observed. Scale bar: 200 μm.

in mucosal glands. Gleason's score was assigned to the prostate obtained from wild-type, heterozygous, and knockout mice treated with DEN. The scores were 1, 3.6 and 7.0 for wild type, heterozygous, and knockout mice, respectively (Table 1). Based on Gleason's score, the wild-type mice's prostate was well differentiated from normal mucosal epithelial cells. The prostate of heterozygous mice with a Gleason's score of 3.6 was classified as well-

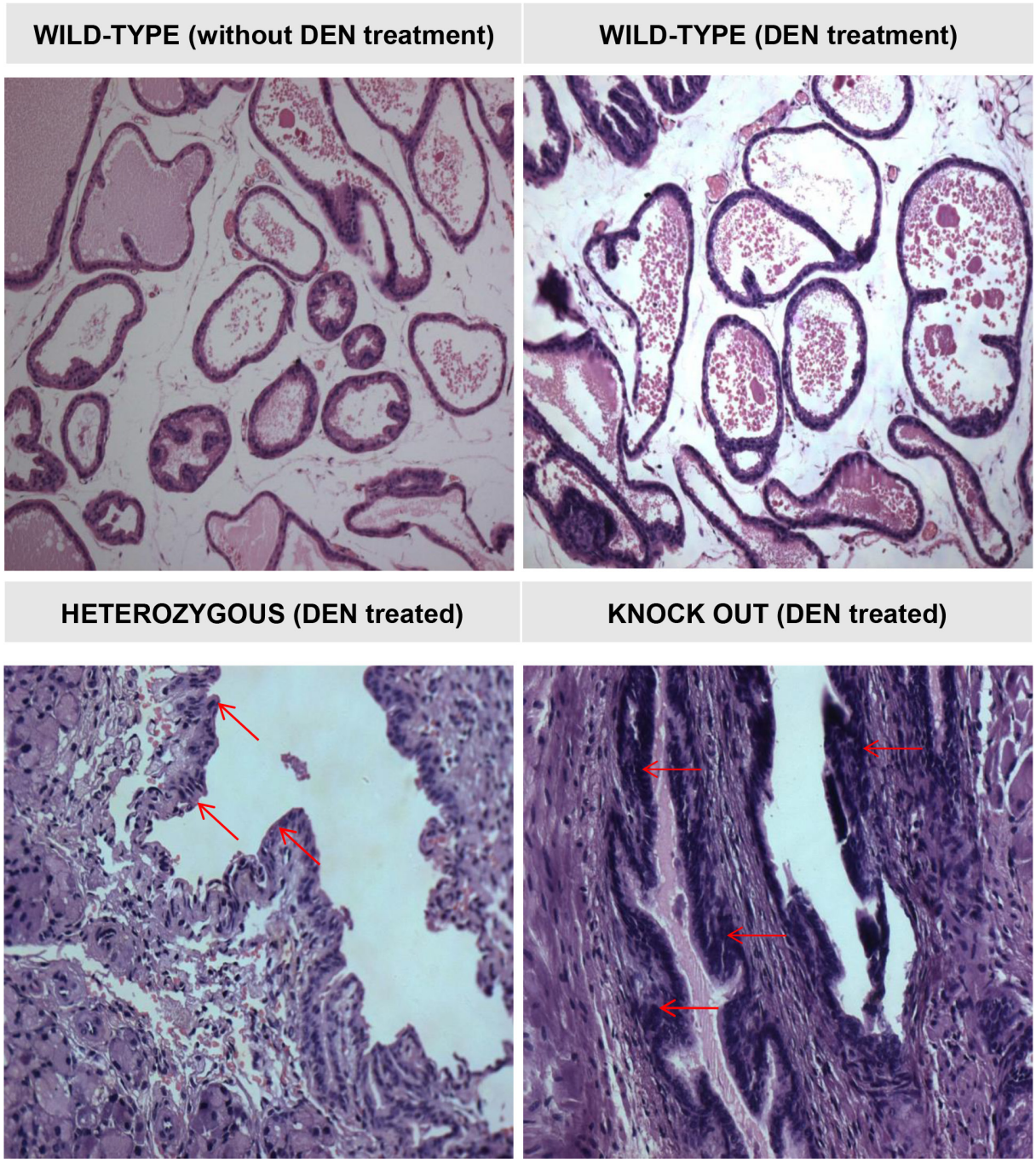


Figure 4. Histopathological changes in the prostate of DEN-treated wild-type, heterozygous, and knockout mice. Prostate from mice treated with DEN were fixed, and 5-micron sections were taken and stained with hematoxylin and eosin. Photomicrographs were recorded in a phase contrast microscope. Arrows indicate the site of histopathological changes observed. Scale bar: 200 μm.

Table 1. Gleason score for the prostate obtained from DEN treated mice.

Genotype	Gleason's score	Histological changes
Wild-type	1	Well differentiated , normal mucosal epithelial cells
Heterozygous	3.6	Well differentiated, moderate anaplastic
Knock-out	7	Markedly anaplastic, poorly differentiated

differentiated with moderate anaplasticity. In the prostate of *Spag11a* knockout mice, which had a Gleason's score of 7.0, the tissue was poorly differentiated and severely anaplastic (Table 1).

4. Discussion

Proteins containing signal peptides are characterised as being secreted and targeted to intracellular or extracellular locations. Proteins produced by the epididymis are diverse in nature, and some contain the signal peptide. The presence of a signal peptide in the SPAG11A protein allows it to be secreted and may have an influence on other organs. Our previous studies demonstrated the effect of ablation of SPAG11A on the susceptibility of male reproductive organs to chemically induced tumorigenesis[10,13]. To provide further evidence, in this study we used *Spag11a* knockout mice to determine whether the cauda, testis, prostate and seminal vesicles are further sensitised to DEN-induced tumorigenesis. We observed that these organs obtained from knockout mice were highly susceptible to tumorigenesis when compared to those obtained from the wild-type mice.

The effect of gene knockout on the histopathological aspects of male reproductive organs has been demonstrated earlier. Dilated lumina of seminiferous tubules were evident in the testis of Vps13a (which encodes a lipid transfer protein, VSP13A) knockout mice[14]. In Odad3 knockout mice, a significant reduction in luminal diameter was observed[15]. Testes and vas deferens of Atp10A gene knockout mice displayed histopathological abnormalities[16]. Testicular dysgenesis, characterised by disorganised seminiferous tubules, occurred in *Pbx1* knockout mice[17]. While the effect of a gene knockout on the altered anatomical structures of the testis is frequently reported, such reports in the other organs of the male reproductive system are scarce. Further, the susceptibility of reproductive organs to chemically induced tumorigenesis is also not well reported. In our recent study, we demonstrated that knockout of the *Spag11a* gene rendered the caput epididymis more susceptible to DEN-induced tumorigenesis, with concomitant alterations in the transcriptome that are indicative of molecular changes that favour cell proliferation[11]. The histological changes observed in the cauda, testis, seminal vesicles, and prostate of DEN-treated heterozygous and knockout mice clearly indicated the pathological state of hyperplasia and inflammation, which is commonly observed in tumorigenesis. Surprisingly, we observed that the prostate was the most affected organ among all the tissues analysed. This observation indicates the role of the SPAG11A protein in prostate function and maintenance. Since no studies have analysed the susceptibility of male reproductive organs to chemically induced tumorigenesis, it becomes increasingly complex to compare the results obtained in this study. It will be interesting to conduct molecular analyses to determine how SPAG11A, a secreted caput epididymis-specific protein, acts on the other organs of the male reproductive tract and affects their function.

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