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# Abnormal spermatogenesis and reduced fertility in transgenic mice expressing the immediate-early protein IE180 of pseudorabies virus

Yukiko Tomioka [a], Masami Morimatsu [a,*], Satoshi Taharaguchi [b], Sayo Yamamoto [c], Haruka Suyama [c], Kinuyo Ozaki [c], Naoki Iwamori [c], Etsuro Ono [c,*]

[a] Division of Disease Model Innovation, Institute for Genetic Medicine, Hokkaido University, Sapporo 060-0815, Japan
[b] Laboratory of Infectious Disease, School of Veterinary Medicine, Azabu University, Kanagawa 252-5201, Japan
[c] Department of Biomedicine, Center of Biomedical Research, Research Center for Human Disease Modeling, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan

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ABSTRACT

Transcription factors of alphaherpesviruses not only control the expression of their own viral genes, but also influence the gene expression of mammalian cells. In the course of breeding of the transgenic mouse line (TgIE96) expressing the immediate-early protein IE180 of pseudorabies virus belonging to the subfamily *Alphaherpesvirinae*, we found that TgIE96 male mice suffered from severe breeding difficulties. Testes of TgIE96 were smaller than that of non-transgenic littermates and abnormal spermatogenesis such as morphological, numerical and functional anomalies of spermatozoa were found in the transgenic mouse line. Expression of IE180 was detected in the germ cells at all stages, especially spermatocytes, and fewer Sertoli cells. In addition, expression of IE180 was also detected in the germinal cells of C57BL/6 mice inoculated with PRV into their testes. These results suggest that IE180 of PRV induces male infertility by abnormal spermatogenesis, which effect morphological, numerical, and functional anomalies of spermatozoa, in transgenic mice.



## 1. Introduction

Infertility is one of the major medical problems worldwide. Approximately 10% of couples expecting the pregnancy are infertility, and the about half is due to male deficiency [1,2]. Most of the male infertility arise from spermatogenic failure, which is the numerical and/or functional abnormality of sperms. It is assumed that many factors including inflammation, infection, high temperature, ischemia, heavy metals, drugs, alcohol, and smoking, negatively affect mammal spermatogenesis. Viral infections are also considered a risk factor in male infertility. Especially, Herpesviridae such as herpes simplex virus (HSV), cytomegalovirus, human herpes virus, were detected in the semen of the male infertility patient in the previous studies, but it is still controversial whether such a viral infection is involved in male infertility [3–6].

Pseudorabies virus (PRV) is classified into the genus *Varicellovirus* of subfamily *Alphaherpesvirinae* same as HSV [7]. PRV invades and spreads along the trigeminal pathway of neonatal pigs, i.e., the nasal mucosa, trigeminal ganglion, trigeminal nuclei, and their projecting areas, such as the cerebellum and thalamus [8]. It causes severe neurological disorders in infected piglets and other domestic, wild, experimental animals. In older latent-infected pigs, the disease is characterized by symptoms respiratory disorders and reproductive failures, such as abortion, mummifications and stillbirths [9,10]. In the mouse infection model, PRV induces acute encephalitis similar to that in piglets [11]. However, latent-infected mouse models, which are generated by immunization or inoculation of the attenuated viral strain, show decreased reproductive performance [12,13].

PRV expresses an immediate-early (IE) protein, IE180, consisting of 1460 amino acid residues. IE180 exhibits a high degree of homology with the IE protein of other alphaherpesvirus, i.e., ICP4 of HSV. IE180 activates the transcription of later viral genes and represses the synthesis of its own RNA [14,15]. Transcription factors of alphaherpesviruses not only control the expression of their own viral genes, but also influence the gene expression of other viruses and mammalian cells [16–18]. Also IE180 is known to be a strong transactivator of several promoters, including other viral and cellular genes [15,19]. Previously, we had hypothesized that the expression of IE180 in host animals would develop neurological abnormalities without viral infection and replication, by

* Corresponding authors. Fax: +81 11 706 7547 (M. Morimatsu), +81 92 642 6165 (E. Ono).
*E-mail addresses:* ytomio@igm.hokudai.ac.jp (Y. Tomioka), mmorimat@igm.hokudai.ac.jp (M. Morimatsu), tsatoshi@azabu-u.ac.jp (S. Taharaguchi), ysayo@anim.med.kyushu-u.ac.jp (S. Yamamoto), sharuka@anim.med.kyushu-u.ac.jp (H. Suyama), k-ozaki@anim.med.kyushu-u.ac.jp (K. Ozaki), iwamori@anim.med.kyushu-u.ac.jp (N. Iwamori), etsuro@anim.med.kyushu-u.ac.jp (E. Ono).

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perturbing gene expression of the host cells. To verify this, we generated transgenic mouse lines expressing IE180, and showed that IE180 expression results in disorganization of the cerebellar cortex and eye [20–22]. In the course of breeding the transgenic mouse line (TgIE96), we found that TgIE96 male mice had small testes and suffered from severe breeding difficulties. Therefore we hypothesized that the IE180 of PRV induced male infertility due to the testes abnormality in transgenic mice, and carried out morphologic and functional analyses of TgIE96 testes in the present study. Here we show that expression of an IE protein of alphaherpesviruses induces male infertility associated with abnormal spermatogenesis, i.e. morphological, numerical, and functional anomaly of spermatozoa, in transgenic mice.

## 2. Methods and materials

### 2.1. Transgenic animals

Ten- to twenty-week-old male PRV IE180 transgenic mice line (TgIE96) and non-transgenic littermates (non-Tg) were used, which were generated previously [20]. The FTA elute micro cards (Whatman) were used for isolation of genomic DNA for genotyping. Transgenes were detected by PCR analysis using specific primers, as described previously [20]. All mice were maintained in the animal facility at our institute and treated according to Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology in Japan.

### 2.2. Sperm count and in vitro fertilization

*In vitro* fertilization (IVF) was carried out in the usual manner. Briefly, TgIE96 or non-Tg sperm from cauda epididymis were preincubated in FERTIUP medium (Kyudo) for 60 min at 37 °C with 5% $CO_2$. Then sperms counts were determined using a hemocytometer and an aliquot of them were added to the drop of CARD MEDIUM (Kyudo) containing the cumulus–oocyte complex from female C57BL/6 mice (final sperm concentration: 200/μl). After 3–4 h, the inseminated oocysts were washed in drops of M16 (Sigma) and cultured for 24 h at 37 °C with 5% $CO_2$. Twenty-four hours after insemination, fertilization rates were determined as the percentage of 2-cell stage embryos.

### 2.3. Antibodies

Polyclonal antibodies against IE180 produced previously in the rabbit were used at the concentration of 1 μg/ml for immunohistochemistry [22]. We also used anti-single stranded DNA (ssDNA) antibody (1:400; Dako) that recognizes DNA fragmentation for detection of apoptotic cells.

### 2.4. Pathological and immunohistochemical analyses

Under deep pentobarbital anesthesia, mice were perfused transcardially with 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer (PB). The testes and epididymides were excised and further immersed overnight in the same fixative or Bouin's fixative. The fixed organs were dehydrated and then embedded in paraffin-wax in the usual manner. Paraffin sections (2–4 μm in thick) were cut and stained with periodic acid-Schiff (PAS) staining. The seminiferous tubular diameters were measured at the central region of the widest cross-section. Immunoperoxidase staining by the streptavidin–biotin method was performed using a Histofine SAB-PO(R) kit (Nichirei) and 3,3′-diaminobenzidine tetrahydrochloride (DAB) detection.

### 2.5. Spermatozoa immunofluorescence

The cauda epididymides were excised from TgIE96 and non-Tg, and isolated spermatozoa were suspended into 0.5 ml M2 medium (Sigma) and incubated for 60 min at 37 °C with 5% $CO_2$. Aliquots of suspension were put onto glass slides, air-dried and fixed in methanol. Spermatozoa on the glass slides were triple-stained with PNA-FITC (Sigma), Mitotracker Red CMX Ros (Invitrogen), and 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Invitrogen). Images were taken by using a fluorescence microscope equipped with CCD camera (Olympus).

### 2.6. Scanning and transmission electron microscope

Ultrastructural analyses of spermatozoa from TgIE96 and non-Tg were performed using transmission electron microscopy (TEM) and scanning electron microscopy (SEM). For TEM, sections of cauda epididymides were fixed with 2% PFA-2.5% glutaraldehyde (GA) in 0.1 M PB and ultrathin sections were prepared and stained with 2% uranyl acetate according to standard procedures. For SEM, spermatozoa from cauda epididymides were preincubated in human tubal fluid medium for 60 min at 37 °C with 5% $CO_2$ and centrifuged at low speed. The sperm pellets were fixed by suspending in 2% PFA, 2% GA in 0.1 M cacodylate buffer. After the fixation, the samples were post-fixated and dehydrated in the usual manner, followed by tert-butyl alcohol freeze-drying method.

### 2.7. PRV infection in mice

PRV YS-81 strain was used for experimental infections. Eight- to ten-week-old male C57BL/6 mice under pentobarbital anesthesia were inoculated in the testes with $10^5$ PFU of virus, diluted to a total volume of 10 μl in PBS. Mice were similarly inoculated with the same volume of PBS as negative control. Genomic DNA was isolated from various tissue including testis and brain by a routine procedure, and IE180 DNA was detected by PCR analysis, as same as described above. Immunohistochemical analyses using anti-IE180 antibody were also performed by the routine procedure mentioned above. All mice were maintained in the animal facility at our institute as mentioned above.

### 2.8. Statistics

The values were expressed as the averages of at least three mice ± standard deviations. Statistical analysis was performed by Student's *t*-test.

## 3. Results

### 3.1. Decrease in testes size and reduced fertility in TgIE96 mice

Grossly, TgIE96 testes were smaller than non-Tg testes as shown in Fig. 1A and their weights were about 60% of non-Tg testes (Fig. 1B). Production of spermatozoa in TgIE96 mice reduced as the number of epididymal sperm was also reduced about 60% of those of non-Tg (Fig. 1C). Motility of sperms collected from the TgIE96 epididymis was obviously poorer than those of non-Tg. Since TgIE96 mice were rarely born in natural mating, IVF was routinely performed for maintaining the transgenic mouse line. IVF using same number of sperms showed that TgIE96 mice exhibited a significant reduced fertilization rate (52%) as compared with that in

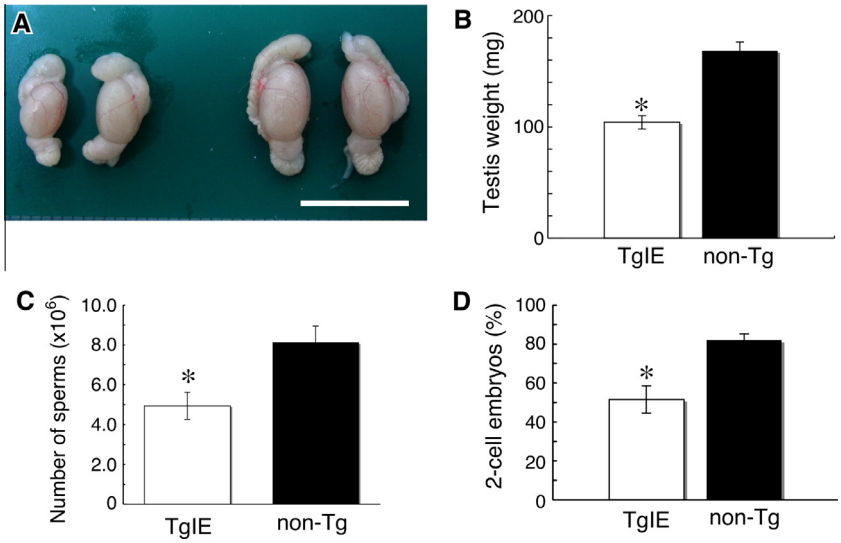


**Fig. 1.** TgIE96 mice exhibit reduction in testes size and weight, numbers of sperms, and fertility. (A) Testes and epididymides from TgIE96 (left) and non-Tg (right) mice at the age of 12 weeks. Bar, 10 mm. (B) Testis weights of TgIE96 and non-Tg mice at the age of 14 weeks ($n = 5$). *$P < 0.0001$. (C) Sperm count of TgIE96 and non-Tg mice ($n = 4$). *$P = 0.0069$. (D) Percentage of 2-cell stage embryos after 24 h following IVF. TgIE96 and non-Tg mice ($n = 4$). *$P < 0.0001$.

non-Tg (82%) (Fig. 1D, $P < 0.0001$). These results suggest that infertility in TgIE96 mice is due to not only numerical but also functional abnormality of sperms.

### 3.2. Abnormal spermatogenesis in TgIE96 mice

To identify the cause of infertility in TgIE96 male mice, histopathological analyses of the testes were performed. In the central regions of the widest cross-sections of testes, the seminiferous tubules were atrophic overall in TgIE96 mice compared with those in non-Tg (Fig. 2A and B). The seminiferous tubular diameters in TgIE96 mice were approximately 75% of those in non-Tg at the same regions (Fig. 2E, $P < 0.001$). Spermatogenic maturation from spermatogonia to spermatocyte, round spermatid, and elongated spermatid at spermatogenic stage VII in non-Tg was shown in Fig. 2D. Although similar spermatogenesis was observed in TgIE96 at same stage, germ cells in each layer, particularly the numbers of elongated spermatid, were obviously decreased (Fig. 2C).

Then the expression of IE180 was examined in TgIE96 and non-Tg testes by immunohistochemistry for IE180 antibody. In TgIE96 testes, IE180 signals were detected in many nuclei of germ cells at all stages, especially spermatocytes, and fewer Sertoli cells (Fig. 2F), but not in non-Tg testes (Fig. 2G). In the widest cross-sections of testes, although ssDNA-positive apoptotic cells were not much observed in non-Tg testes (Fig. 2I), the number of ssDNA-positive cells was markedly increased in the TgIE96 testes (Fig. 2H). By counting, the number of ssDNA-positive cells in the entire widest cross-sections was significantly increased in TgIE96 mice (Fig. 2J) ($P = 0.004$). When double immunofluorescence for IE180 (green) and ssDNA (red) was applied to TgIE96 testis, ssDNA-positive cells were observed adjacent to the IE180-positive cell population, and their colocalization in the same cells were occasionally detected (Fig. 2K).

Sperms collected from cauda epididymides of TgIE96 were obviously less active than those of non-Tg as described above. To clarify the abnormality of TgIE96 sperms, sperms from cauda epididymides were triple-stained with PNA-FITC, Mitotracker, and DAPI (Fig. 3A). While most sperms have PNA-positive acrosome in non-Tg testes (Fig. 3A, right), PNA-negative sperms were often observed in TgIE96 testes (Fig. 3A, left, Fig. 3B). TEM of cauda epididymides revealed that the deformation of sperm heads and acrosomes were often observed in TgIE96 mice (Fig. 3C and D). The tangled sperm tails as well as deformed sperm heads were marked in TgIE96 mice by SEM analysis (Fig. 3F). Such abnormal sperms were rarely noticed in the non-Tg mice (Fig. 3E and G).

### 3.3. PRV infection in mouse testis

In order to examine whether PRV infects to the germinal cells of mouse testes, PRV YS-81 strain was intratesticularly inoculated into C57BL/6 mice. Viral DNA and IE180 antigens were detected in the testes of C57BL/6 mice inoculated with PRV after at least post-inoculated days 3, when infected mice died with neurological symptom (Fig. 4). PRV DNA was continually identified in the testes from post-inoculated day 1 up to day 3 (Fig. 4A). In the brain, viral DNA began to be detected from post-inoculated day 2 or day 3. Immunohistochemical analysis performed on the testes revealed that IE180 antigens were detected in the germinal cells (Fig. 4C), especially spermatocytes, as well as Leydig cells and other cells in the interstitial tissue (Fig. 4B). These results support a possibility that the expression of IE180 in the germinal cells may induce abnormal spermatogenesis in the testes infected with PRV.

## 4. Discussion

In the present study, abnormal spermatogenesis was found in the transgenic mouse line expressing IE180 (TgIE96). In our previous studies, it was also reported that transgenic mouse lines expressing IE180 showed cerebellar and ocular abnormalities [20–22]. These findings suggest that IE180 affects host development without active viral replication and invasion, by disordering gene expression of the host cells. IE180 contains three important functional domains, the acidic transcriptional active domain, the DNA-binding domain, and the nuclear localization signal, and retains the transactivation and autorepression potentials [23–26]. The previous results suggested that all three functional domains of IE180 were necessary for manifestation of the cerebellar and ocular abnormalities, through the analyses of transgenic mouse lines expressing IE180 and its truncated mutants [22]. Because the reproductive disorder was also found in the transgenic mouse line expressing the intact IE180 rather than its truncated mutants,

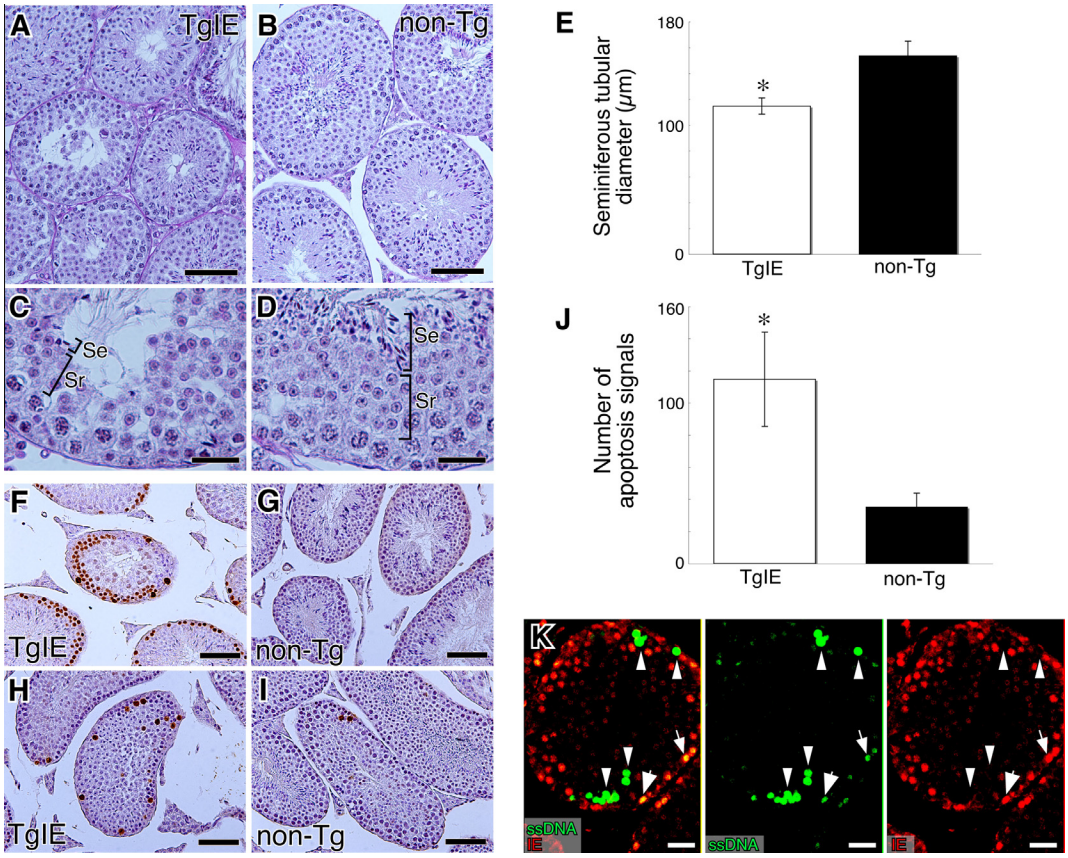


**Fig. 2.** TgIE96 mice exhibit abnormal spermatogenesis in testes. (A–D) Histopathological findings of testes from TgIE96 (A and C) and Non-Tg (B and D) mice. The central region of the widest cross-section of testes (A and B) and the high magnification of seminiferous tubules at spermatogenic stage VII (C and D). Layers of round spermatid (Sr) and elongated spermatid (Se). PAS staining. Bars, 50 μm (A and B); 20 μm (C and D). (E) The average of seminiferous tubular diameters. $^{*}P < 0.001$. (F–I) Immunohistochemical analyses of TgIE96 (F and H) and non-Tg (G and I) mice, for IE180 (F and G) and apoptosis marker ssDNA (H and I). Bars, 50 μm. (J) The number of ssDNA-positive cells per the widest cross-section of testes. Significant differences between TgIE96 and non-Tg mice ($^{*}P = 0.004$, $n = 3$). (K) Double immunofluorescence for ssDNA (green) and IE180 (red). Bars, 50 μm. Arrowheads: ssDNA-positive, IE180-negative cells. Arrows: colocalization of ssDNA and IE180. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

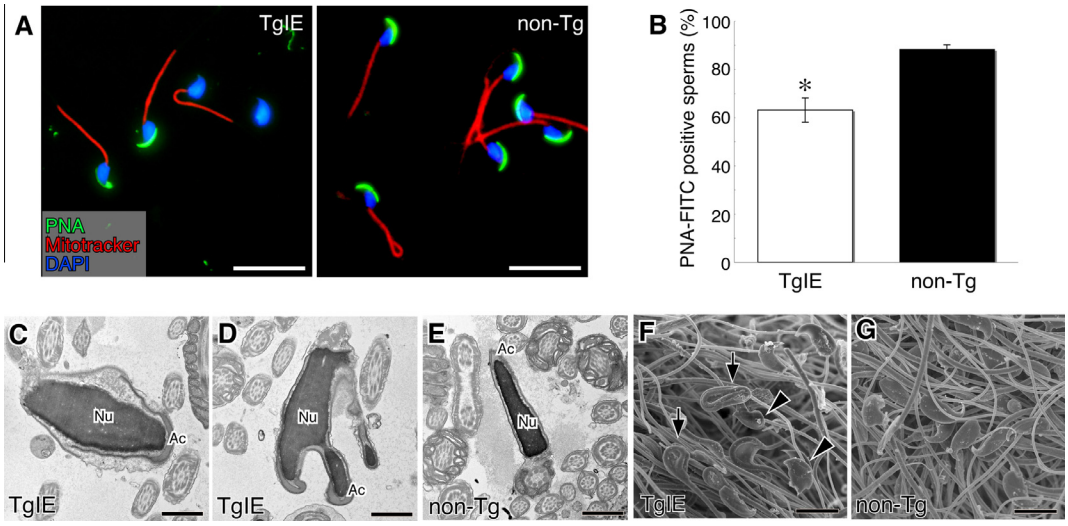


**Fig. 3.** TgIE96 sperms from epididymides exhibit morphological anomaly. (A) Triple-immunofluorescence staining of sperms. PNA-FITC (green), Mitotracker (red), and DAPI (blue). Bars, 40 μm. (B) Percentage of PNA-positive sperms from TgIE96 and non-Tg mice. Significant decrease of PNA-positive sperms in TgIE96 ($^{*}P = 0.006$). TEM of cauda epididymides of TgIE96 (C and D) and non-Tg (E) mice. Bars, 1 μm. Nu: spermatozoal nuculeus, Ac: acrosome. SEM of spermatozoa from cauda epididymides of TgIE96 (F) and non-Tg (G). Bars, 5 μm. Arrows: tangled sperm tails. Arrowheads: deformed sperm heads. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

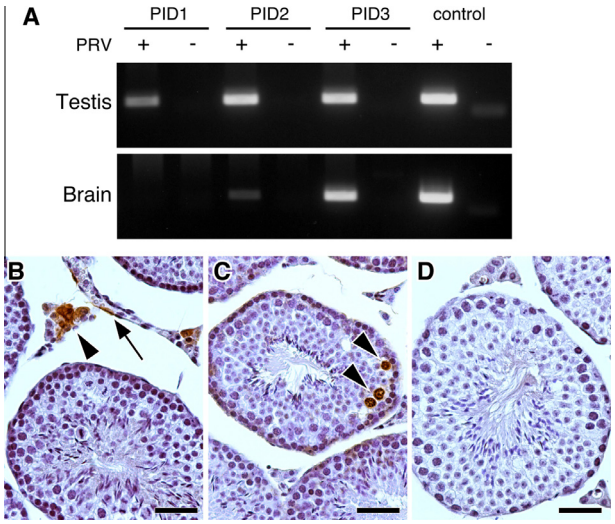


**Fig. 4.** Detection of viral IE180 DNA and antigen in the mice inoculated with PRV YS-81 strain into testes. (A) PCR detection of IE180 DNA. PID1, 2, 3: post-inoculated days 1, 2, 3; PRV+: PRV inoculated, PRV-: PBS inoculated; control: PRV-inoculated cellular DNA. (B–D) Immunohistochemical detection of IE180 in the Leydig cells (B, arrowhead), vascular endothelium (B, arrow), and germ cells (C, arrowheads). Testes from the mice at 3 days post-inoculated with PRV (B and C) or PBS (D). Bars, 50 μm.

it was suggested that the intact IE180 as the multifunctional transcription factor was also necessary for disturbing spermatogenesis.

It has been reported that thymidine kinase of HSV (HSV-TK) accumulation induced male infertility by spermatogenesis disruption and apoptosis of germ cells [27–29]. Histological findings suggesting Sertoli cell-mediated ablation of germ cells were prominently found in transgenic rodents expressing HSV-TK [27,29], although those findings were not observed in TgIE96. No spermatozoon was observed in the epididymis of 6-month-old HSV-TK transgenic rodents, whereas fertilizable sperms were obtained from TgIE96 similar in age, in spite of the strong IE180 expression (data not shown). Therefore, male infertility of TgIE96 might depend on a mechanism different from that of HSV-TK transgenic rodents. There must be an indication that these transgenic models cannot reproduce the influence of alphaherpesvirus infection in the germinal cells, but only show the toxicity of these viral proteins in spermatogenesis. However, considering that the alphaherpesvirus infection is characterized by the latency and reactivation, and these viral proteins may be intermittently expressed in testis, we believe that the association between these viral protein and reproductive performance should not be excluded and further examination should be carried out about the mechanisms of the abnormal spermatogenesis in these transgenic mice to better understanding of the male infertility associated with alphaherpesvirus infection.

There were several reports that the presence of HSV DNA in human spermatozoa decreased semen quality such as poor sperm count and reduced motility [3–5]. As regards PRV, it was reported that testicular degeneration and transient elevation in the number of abnormal sperms were observed in boars experimentally infected by the intratesticular route [30,31]. In addition, there is a report that male mice intraperitoneally challenged with attenuated strain of PRV showed decreased reproductive performance [13]. The present results showed that PRV could infect germ cells in the mouse testis. Taken together, these findings support the hypothesis that male genital infection with alphaherpesviruses including PRV may affect spermatogenesis.

HSV ICP4, which is an IE180 homolog, has been proposed to have the anti-apoptotic functions [32,33]. In the present study, IE180 seemed to have apoptotic effect rather than anti-apoptotic, at least in the TgIE96 testes. Though it has not been yet revealed whether IE180 has the anti-apoptotic or apoptotic effect, it has been reported that IE180 suppresses phosphorylation of translation initiation factor eIF2α [34]. Because it is suggested that phosphorylated eIF2α can have both roles of cytoprotection and apoptosis induction depending on the stress conditions [35,36], further examination should be performed to clarify the role of IE180 in the apoptotic responses of the hosts.

In summary, we conclude that IE180 of PRV induces male infertility by abnormal spermatogenesis, which effect morphological, numerical, and functional anomaly of spermatozoa, in transgenic mice. The present findings suggest the possibility that PRV IE180 affects spermatogenesis without active viral replication and invasion, by perturbing gene expression of the host cells, and might extend our understanding on function of viral protein in the herpesvirus-infected male infertility.

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