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## Original Article

# Expression profile of spermatogenesis associated genes in male germ cells during postnatal development in mice

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**ABSTRACT**   Spermatogonial stem cells are self-renewal and differentiate into sperm in post-pubertal mammals. There exists a balance between the self-renewal and differentiation in the testes. Spermatogonial stem cells make up only 0.03% of testicular cells in adult mice. These cells maintain sperm production by differentiating after puberty. Therefore, analyzing the expression of genes associated with spermatogenesis is critical for understanding differentiation. The present study aimed to establish the postnatal period of cells in relation to spermatogenesis. To study the expression of differentiated and undifferentiated marker genes in enriched spermatogonial stem cells, *in vitro* culture was performed and cells from pup (6–8-day-old) and adult (4-months-old) testicular tissues were isolated. As a result, undifferentiated genes, *Pax7, Plzf, GFRa1, Etv5* and *Bcl6b*, were highly increased in cultured spermaotogonial stem cells compared with pup and adult testicular cells. On the other hands, differentiated gene, *c-kit* was highly increased in adult testicular cells, Also *Stra8* gene was highly increased in pup and adult testicular cells. This study provides a better understanding of spermatogenesis-associated gene expression during postnatal periods.

**Keywords:** adult testicular cells, gene expression, pup testicular cells, spermatogenesis, spermatogonial stem cells

## INTRODUCTION

Spermatogonial stem cells (SSCs) are the foundation of spermatogenesis in the testes and are essential for male fertility (Phillips et al., 2010; Park et al., 2014). SSCs have two major roles; first, they maintain a pool of self-renewing cells, allowing the proliferation of stem cell populations, and second, they support sperm production by the spermatogonial differentiation of SSCs in post-pubertal males (Oatley and Brinster, 2008). SSCs comprise only 0.03% of total germ cells in mice (Phillips et al., 2010). In postnatal mouse testes, SSCs are located on the basement membrane of seminiferous tubules surrounded by Sertoli cells (de Rooij, 1973).

Spermatogenesis appears as dual division; first, $A_{single}$ spermatogonia ($A_s$) is divided into $A_{pair}$ ($A_{pr}$, chain of two), $A_{aligned4}$ ($A_{al4}$, chain of four), $A_{aligned8}$ ($A_{al8}$, chain of eight), and $A_{aligned16}$ ($A_{al16}$, chain of sixteen) through mitosis. Af-

ter differentiation to A1 spermatogonia, A2, A3, A4, intermediate (In), and B spermatogonia as well as meiotic spermatocytes are generated. Meiotic spermatocytes are divided into secondary spermatocytes and round spermatids, which are produced in mature sperm, by secondary meiosis (Valli et al., 2015).

$A_s$ is a function of division to new $A_s$ spermatogonia and maintains an undifferentiated spermatogonia state (de Rooij, 1973). In addition, $A_{pr}$ and $A_{al4}$ self-renew to produce single spermatogonia by complete cytokinesis (de Rooij and Griswold, 2012). Undifferentiated spermatogonia in mouse testes express numerous self-renewal genes, such as paired box 7 (*Pax7*), promyelocytic leukemia zinc finger (*Plzf*), GDNF-family receptor α1 (*GFRa1*), Ets variant gene 5 (*Etv5*), and B-cell CLL/lymphoma 6, member B (*Bcl6b*) (Costoya et al., 2004; Buageaw et al., 2005; Schlesser et al., 2008; Ishii et al., 2012; Aloisio et al., 2014), while proto-oncogene c-kit (*c-kit*) and stimulated by retinoic acid 8 (*Stra8*) are expressed by all differentiated spermatogonia (Yoshinaga et al., 1991; Zhou et al., 2008). The *Pax7* gene is specifically expressed in $A_s$ spermatozoa (Aloisio et al., 2014). The large ETS family of transcription factors, *Etv5*, is important in SSC development. *Etv5* gene deficiency causes loss of all germ cells and the Sertoli cell-only phenotype in mice by 10 weeks after birth (Chen et al., 2005). The *c-kit* gene is expressed in type A, In, and type B spermatogonia. Also, mutated *c-kit* gene is generating loss of melanocyte and germ cells (Sorrentino et al., 1991). The *Stra8* gene is expressed in germ cells from mitosis to meiosis and plays a key role during initial meiosis (Giuili et al., 2002).

In mice, SSCs directly develop to A1 spermatogonia on day 6 after birth, and spermatogenesis is completed during the differentiation of SSCs within 3 weeks in mice (Culty, 2009). Therefore, this study aimed to identify the cells involved in the spermatogenesis period and analyze the gene expression of spermatogenesis-associated marker genes. Therefore, we established a culture of SSCs (enriched undifferentiated SSCs), pup testicular cells (PTCs, pre-puberty), and adult testicular cells (ATCs, post-puberty) for gene expression analysis.

## MATERIALS AND METHODS

### Animals

Male C57/BL6J-TG-EGFP (Jackson Laboratory, Bar Harbor, Maine, USA) and female C57/BL6J mice(Samtako Bio, Osan, Gyeonggi-do, Korea) were used. Six-week-old female mice were obtained separately. All animal experiments were approved by the Institutional Animal Care and Use Committee of Chung-Ang University (no. 2020-00057) and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the NIH. All animals had free access to food and water during the experiments.

### Isolation and culture of mouse spermatogonial stem cells

Mouse SSCs were isolated according to a previously described method (Oatley et al., 2007). Briefly, 6-8-day-old male mice were euthanized using carbon dioxide. Seminiferous tubules were isolated from the testes and washed in DPBS. After treatment with 2:1 (Invitrogen, Carlsbad, CA, USA) of 0.025% typsin-EDTA (Invitrogen, Carlsbad, CA, USA) and 7 mg/mL DNase I (Roche, Basel, Switzerland) at 37℃ for 5 min, single cells were recovered. Filtration was performed using a 40-μm mesh, and centrifugation was then undertaken at 600 × g for 7 min at 4℃. The supernatant was then discarded, and a 30% Percoll gradient was applied to remove the erythrocytes and debris. For the purification of SSCs, the MACS method was used (Oatley and Brinster, 2006) using anti-Thy1 antibody microbeads (1:10, Miltenyi Biotech, Auburn, CA, USA) for 15 min at 4℃. Thy1-positive cells were plated onto mitotically inactivated STO (SIM mouse embryo-derived thioguanine- and ouabain-resistant) feeder cells. SSC culturing was conducted based on a previously reported method (Jung et al., 2020a).

### Isolation of testicular cells

Pup (6-8-day-old) and adult (4-month-old) male mice were euthanized and the testes were obtained. These were then decapsulated and treated with 2:1 (Invitrogen, Carlsbad, CA, USA) of 0.025% typsin-EDTA (Invitrogen, Carlsbad, CA, USA) and 7 mg/mL DNase I (Roche, Basel, Switzerland) at 37℃ for 5 min, and single cells were isolated. Filtration was performed using a 40-μm mesh followed by centrifugation at 600 × g for 7 min at 4℃. The erythrocytes and debris were then removed, and a 30% Percoll gradient was applied followed by centrifugation at 600 × g for 10 min at 4℃. Cell pellets were then resuspended in Trizol reagent (Invitrogen) for cDNA synthesis.

## Hematoxylin & eosin staining

The mouse testes were maintained in 4% formaldehyde overnight at 4℃. The fixed tissue was then embedded in paraffin, and paraffin sections (5 μm) were deparaffinized in xylene and re-hydrated in serially diluted alcohol. The samples were then washed in running tap water for 5 min, incubated in Mayer's hematoxylin solution for 1 min, and washed in running tap water for 20 min. The samples were moved to a jar filled with Eosin solution and incubated for 1 min. After dehydration and clearing, the samples were visualized under a Ni-U microscope (Nikon, Tokyo, Japan). NIS Elements imaging software (Nikon, Tokyo, Japan) was used for analysis.

## Immunohistochemistry

For immunohistochemistry analysis, paraffin sections (5 μm) were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and left for 1 h in blocking solution containing serum. Anti-VASA antibody (Santa Cruz Biotechnology, Dallas, TX, USA) was diluted to 1:100 in PBS and incubated overnight at 4℃. After washing with PBS containing Tween 20, the sections were incubated for 1 h with fluorescent conjugated secondary antibodies and visualized under a fluorescent microscope. DAPI was used for nuclei counterstaining.

## Quantitative reverse transcription polymerase chain reaction (qRT–PCR)

Total RNA was isolated from SSCs and testicular cells using a PureLink™ RNA Mini Kit (Invitrogen, USA), according to the manufacturer's protocol. cDNA was synthesized from 1000 ng of total RNA using a SuperScript® IV First-Strand Synthesis System (Invitrogen) and oligo-(dT) primers, according to the manufacturer's instructions. For qRT-PCR, 5 μL of SYBR Green PCR Master Mix, 1 μL of primers, and distilled water up to 20 μL were used. Each cDNA was used as a template for PCR amplification in combination with designed gene-specific primers (Table 1). The assay was performed in triplicate using a 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) in 96-well plates (Applied Biosystems). qRT-PCR was performed for two-step thermal cycling as follows: 40 cycles of 95℃ for 20 s and 60℃ for 60 s, followed by a melting stage of 95℃ for 15 s, 60℃ for 60 s, 95℃ for 30 s, and 60℃ for 15 s. Expression levels were normalized to the amount of *GAPDH*, and data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

## Statistical analysis

All experiments were repeated at least thrice, and statistical analysis was performed using one-way analysis of variance with Tukey's honestly significant difference test as a post-hoc test, and the significance level was set at $p < 0.05$. The results are expressed as the mean ± SEM of triplicate independent samples.

## RESULTS

### Identification of germ cells in mouse testes

Mouse mitotic and meiotic germ cell markers, Vasa homologs (VASA, also known as DEAE-box helicase (DDX4)), were enriched in primordial germ cells and spermatogenic cells in mice (Toyooka et al., 2000). The VASA protein expression and subcellular localization of testes tissue were assessed in both PTCs and ATCs (Fig. 1A). In addition, qRT-PCR analysis showed significantly higher expression in ATCs compared with cultured SSCs and PTCs (Fig. 1B). These results indicated abundant amounts of germ cells in ATCs compared with cultured SSCs and PTCs.

Table 1. The sequence of primers for qRT-PCR analysis

| Gene | Forward (5'–3') | Reverse (5'–3') |
|------|-----------------|------------------|
| *Vasa* | GAGATTGCCTTCAGTACCTATGTG | GTGCTTGCCCTGGTAATTCT |
| *Pax7* | CTCAGTGAGTTCGATTAGCCG | AGACGGTTCCCTTTGTCGC |
| *Plzf* | CACCTTCGCTCACATACAGG | ACTTCTTGCCACAGCCATTAC |
| *GFRa1* | GTGTGCAGATGCTGTGGACT | TTCAGTGCTTCACACGCACT |
| *Etv5* | CCCGGATGCACTCTTCTCTATG | TCGGATTCTGCCTTCAGGAA |
| *Bcl6b* | TACTTCAAGGCTTCGCCTCTCT | CTACGTGTTCCATCTGCAAATAGG |
| *c–kit* | AGAAGCAGATCTCGGACAGC | CATCACAGAAGCCAGAAGGAC |
| *Stra8* | GTTTCCTGCGTGTTCCACAAG | CACCCGAGGCTCAAGCTTC |
| *Gapdh* | CTGACGTGCCGCCTGGAGAA | CCCCGGCATCGAAGGTGGAA |
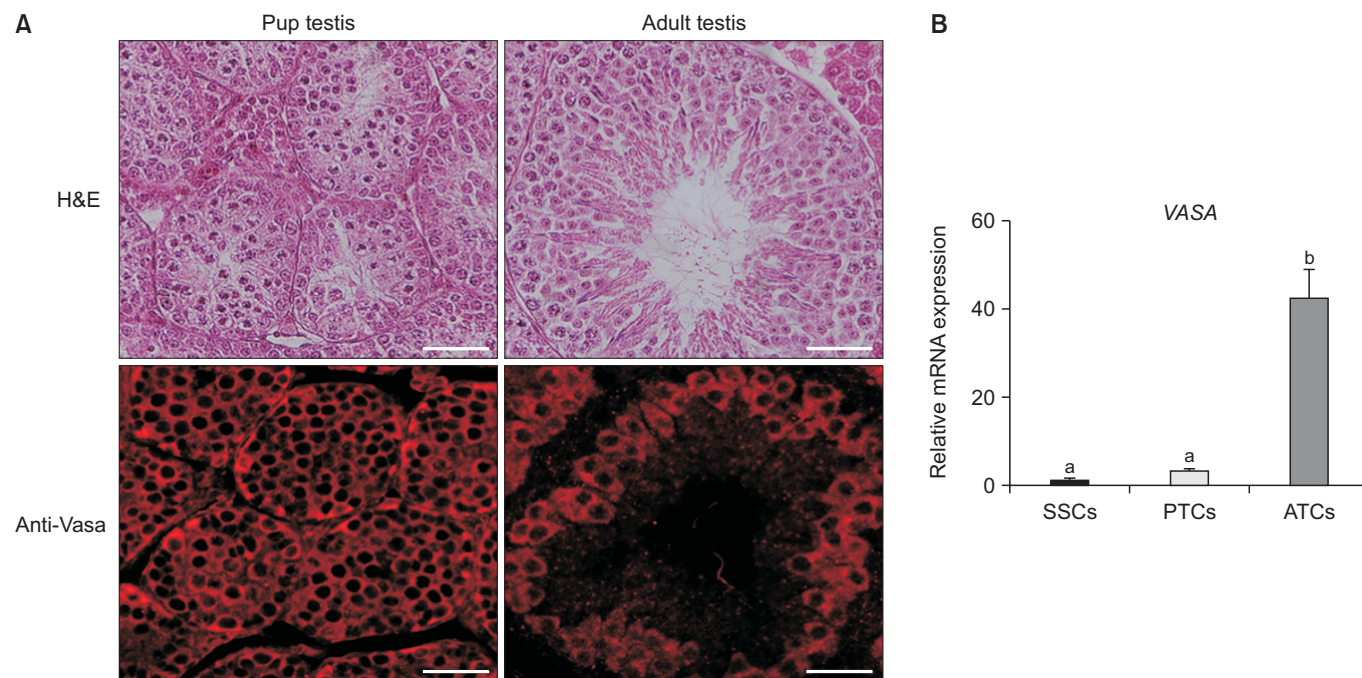
**Fig. 1.** Expression of Vasa in pup and adult testes. (A) Detection of pup and adult testis morphology by H&E staining. The pup testes had smaller seminiferous tubules than the adult testes. The Vasa protein was expressed in cytoplasm of both pup and adult testes. Scale bar = 100 μm. (B) Analysis of *Vasa* gene expression using qRT-PCR. The *Vasa* gene was highly expressed in adult testicular cells (ATCs) compared with cultured spermatogonial stem cells (SSCs) and pup testicular cells (PTCs). These data were normalized by *Gapdh*, and the error bars represent ± SEM for three independent replicates. Different letters are statistically significant ($p < 0.05$).

## Differential expression of undifferentiated spermatogonial stem cell–associated genes

qRT-PCR was performed to analyze the expression of undifferentiated SSC-related genes in cultured SSCs, PTCs, and ATCs (Fig. 2). The expression of undifferentiated SSC marker genes *Pax7*, *Plzf*, *Gfra1*, *Etv5*, and *Bcl6b* showed a significant increase in SSCs (enriched undifferentiated SSCs).

## Differential expression of differentiated spermatogonial stem cell–associated genes

Additionally, the expression of differentiation-related marker genes, such as *c-kit* and *Stra8*, was verified (Fig. 3). *c-kit* gene expression was significantly increased in ATCs compared to in SSCs and PTCs. However, there was no significant difference between SSCs and PTCs. The *Stra8* gene was highly expressed in PTCs and ATCs compared to in SSCs.

## DISCUSSION

This study confirmed gene expression profiling of rela-tive spermatogenesis during postnatal periods. Three types of cells were used to identify gene expression. qRT-PCR analysis showed high expression of undifferentiated genes in SSCs. However, lower expression levels of differ-entiated genes were found compared with PTCs and ATCs. SSCs were isolated from pup testes tissue and sorted using Thy1 antibody to obtain an enriched cell population for SSCs. In many previous studies, Thy1 was used to sepa-rate the mouse SSCs (Karmakar et al., 2017; Jung et al., 2020b). In addition, it was found that the *Pax7* gene was expressed at higher levels in SSCs than in PTCs. The *Pax7* gene is limited to $A_s$ undifferentiated SSCs (Aloisio et al., 2014). Therefore, the population of undifferentiated $A_s$ spermatogonia was enriched. In addition, the transcrip-tion factor gene *Plzf* (also known as *ZBTB16*) is involved in the regulation of self-renewal and the maintenance of stem cells (Costoya et al., 2004). It is expressed by all undifferentiated $A_s$, $A_{pr}$, $A_{al4}$, $A_{al8}$, and $A_{al16}$ spermatogonia. However, the *GFRa1* gene is expressed only in $A_s$, $A_{pr}$, and $A_{al4}$ spermatogonia (Hara et al., 2014), and plays an important role in undifferentiated SSCs. Here, our results showed that *Plzf* was not significantly expressed between
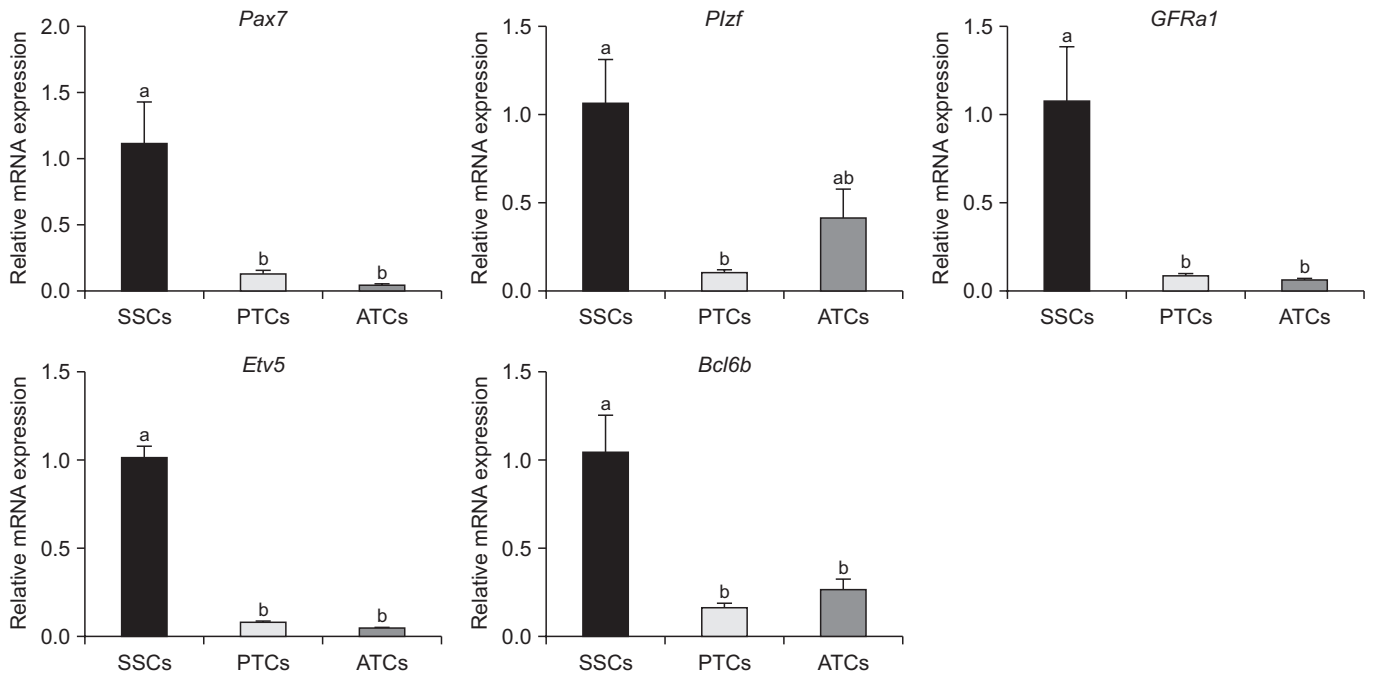
**Fig. 2.** Analysis of undifferentiated spermatogonial stem cell (SSC)-associated gene expression using qRT-PCR. A$_{single}$ (A$_s$) specific expression gene, *Pax7*, was highly expressed in cultured SSCs. Additionally, the expression of undifferentiated marker genes *Plzf*, *GFRa1*, *Etv5*, and *Bcl6b* was significantly increased in SSCs compared with pup testicular cells (PTCs) and adult testicular cells (ATCs). These data were normalized by *Gapdh*, and the error bars represent ± SEM for three independent replicates. Different letters are statistically significant ($p < 0.05$).
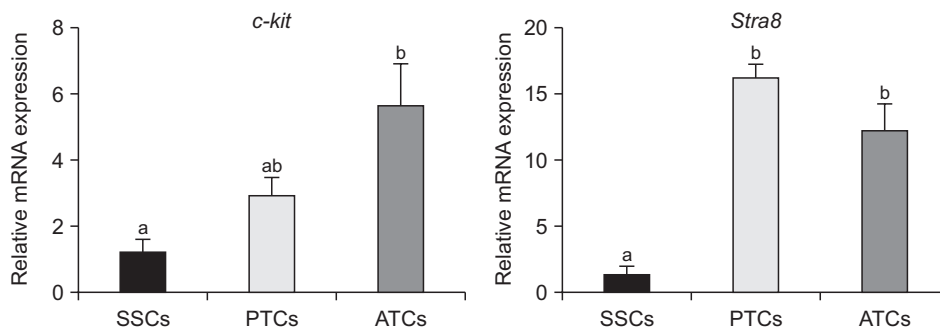


**Fig. 3.** Analysis of differentiated spermatogonial stem cell (SSC)-associated gene expression using qRT-PCR. The expression of undifferentiated marker genes *c-kit* and *Stra8* was significantly increased in pup testicular cells (PTCs) and adult testicular cells (ATCs) compared to in SSCs. These data were normalized by *Gapdh*, and the error bars represent ± SEM for three independent replicates. Different letters are statistically significant ($p < 0.05$).

SSCs and ATCs. The results also indicated that *GFRa1* was more appropriate than *Plzf*, which uses undifferentiated markers. Both Etv5 and Bcl6b are associated with glial cell line-derived neurotrophic factor (GDNF)-regulation, self-renewal, and proliferation in SSCs (Oatley et al., 2006; Wu et al., 2011). These genes were highly expressed in SSCs, such as the GFRa1 gene. Therefore, it is a useful marker for the detection of undifferentiated SSCs.

Auharek and França (2010) reported that 5-day-old male mice consist of approximately 20,000 undifferenti-

ated spermatogonia and an extremely small population of differentiated spermatogonia (Auharek and de França, 2010). In contrast, 100-day-old male mice have a similar undifferentiated spermatogonia cell population with young-aged mice but have more than 400,000 cells of differentiated spermatogonia. The PTCs were isolated from pup testes without sorting by Thy1, which consists of whole testicular cells. As a result, PTCs appeared to have lower expression of undifferentiation genes but higher expression of differentiation genes. We found that
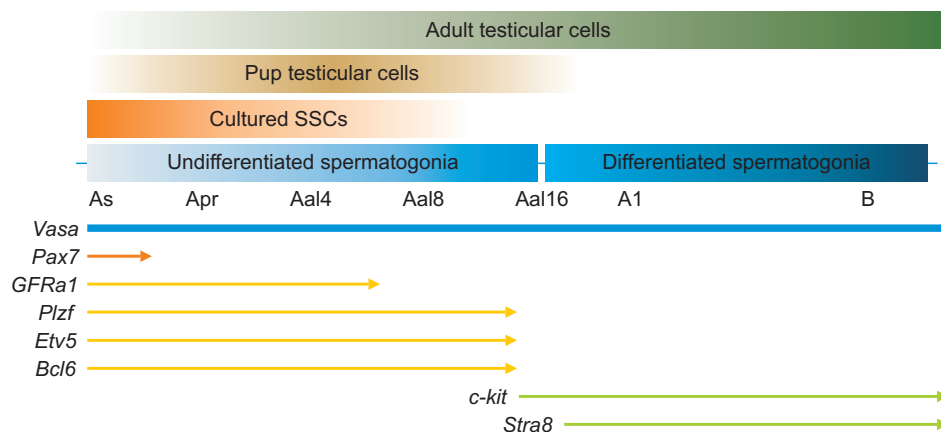
**Fig. 4.** Schematic overview of the gene expression analysis of spermatogenesis-associated genes in cultured spermatogonial stem cells (SSCs), pup testicular cells (PTCs), and adult testicular cells (ATCs).

the PTCs contained more differentiated cells than undifferentiated cells compared with cultured SSCs (germ cells enriched for SSCs through *in vitro* culture). The *c-kit* gene is involved in differentiating $A_{al}$ and $A_1$ spermatogonia, which differentiate from $A_{al}$ to $A_1$ spermatogonia (Yoshinaga et al., 1991; Schrans-Stassen et al., 1999). These results suggest that PTCs may be used as an indication of the middle stage between $A_{al}$ and $A_1$ spermatogonia. Retinoic acid is promoted by the differentiation of undifferentiated spermatogonia, which is stimulated by retinoic acid-8 (*Stra8*) gene, which is involved in the first round of meiotic spermatogenesis (Li et al., 2011) and is expressed in preleptotene spermatocytes (Zhou et al., 2008). Our results indicated that the differentiated marker *Stra8* was higher expressed in PTCs and ATCs than in cultured SSCs.

In conclusion, this study demonstrated the expression of undifferentiated and differentiated spermatogonia marker genes in postnatal periods. As shown in Fig. 4, we focused on the expression of several marker genes in postnatal periods, including cultured SSCs. The results of this study provide the characteristics of, and aid in the understanding of, the various spermatogenesis stages that involve the fate decisions and differentiation of SSCs. In addition, it is a useful tool for the verification of spermatogenesis using spermatogenesis-associated marker genes.

## CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

## AUTHOR CONTRIBUTIONS

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Formal analysis: Jin Seop Ahn
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Project administration: Buom-Yong Ryu
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