

# Expression of Promyelocytic Leukemia (PML) Protein in the Mouse Developing Spermatogonia

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## Research

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# Abstract

**Background:** Promyelocytic leukemia (PML) as the main protein of PML nuclear bodies regulates various physiological processes such as transcription, DNA repair, apoptosis, senescence, and several signaling pathways in different cell types. It is well known that the PML protein is involved in the regulation of stem cell properties by maintaining an open chromatin conformation for the regulatory regions of the Oct4 gene. However, there is no experimental evidence for the presence and function of PML protein in the testis tissue.

**Results:** In this study, we show the presence of PML protein in the developing mouse testis and its co-expression with the OCT4 protein. Immunohistochemical analysis of testis mirror sections shows that PML is co-expressed with the OCT4 protein in the outermost cellular layer of seminiferous tubules, where the spermatogonial stem cells are located.

**Conclusions:** Our findings suggest that the PML protein might be involved in the stemness of spermatogonial stem cells at different stages of its development, even before earning the ability to produce mature sperm.

## Background

Promyelocytic leukemia nuclear bodies (PML NBs), also known as PML oncogenic domain, nuclear domain 10, or Kremer body (Dellaire and Bazett-Jones, 2004; Wang et al., 2004) are subnuclear protein structures that are in close association with chromatin fibers (Bazett-Jones et al., 2008; Dehghani et al., 2005a; Eskiw et al., 2004). They represent nuclear multiprotein complexes consisting of PML and additional proteins such as Daxx, sp100, and SUMO-1 (Bernardi et al., 2008; Scherer et al., 2016). The expression of the PML gene is essential for the formation of PML protein and nuclear bodies. The core proteins of PML NBs are PML proteins that are generated by alternative splicing of the PML gene as different isoforms. These isoforms are capable of binding and specific interactions with other cellular components (Ivanschitz et al., 2015; Ohsaki et al., 2016). The C termini region of these proteins may be responsible for specific interactions with other cellular components and constrain the subcellular localization of PML protein (Block et al., 2006). The PML protein and PML NBs are dynamic subnuclear compartments that are implicated in several cellular processes (Dellaire et al., 2006a), including gene transcription (both activation and repression), viral pathogenicity, tumor suppression, proteasomal degradation, cellular senescence, apoptosis, DNA repair, and pluripotency (Hadjimichael et al., 2017; Lallemand-Breitenbach and de Thé, 2018).

It has been demonstrated that depending on the cell type, stage of the cell cycle, and environmental conditions, PML NBs vary in size, number, and biochemical composition (Dellaire et al., 2006b, 2006c; Hsu and Kao, 2018; Lallemand-Breitenbach and de Thé, 2010). PML bodies establish a physical connection with the surrounding chromatin fibers, retain their position from a cell cycle to the next cycle, and replicate during cell division (Bazett-Jones et al., 2008; Bernardi and Pandolfi, 2007; Dellaire, 2006;

Dellaire et al., 2006d). These bodies can be the right candidates to function as molecular crowding hubs for the regulation of transcription of active genes and differential gene expression during cell differentiation (Block et al., 2006). Experimental evidence shows that PML is involved in the regulation of stem cell properties by maintaining an open chromatin conformation for the promoter region of the Oct4 gene (Chuang et al., 2011). It is well known that the OCT4, SOX2, NANOG, alkaline phosphatase, protein kinase C, and other proteins, lncRNAs, and chromatin organization are important for stemness in different physiological and cancer states (Ahmed et al., 2010; Chuang et al., 2011; Dehghani and Hahnel, 2005; Dehghani et al., 2000, 2005b; Efroni et al., 2008; Es-haghi et al., 2016; O'Connor et al., 2008; Zare et al., 2018). Dann et al. specifically investigated the role of OCT4 in spermatogonial stem cell (SSC) self-renewal (Dann et al., 2008). Thus, the presence of PML NBs in spermatogonial stem cells might be critical for the maintenance of stemness in these cells.

In the previous study, we have shown that mature sperm and oocyte cells do not contain PML nuclear bodies (Ebrahimian et al., 2010). However, these bodies appear in the developmental stages of the newly formed mouse embryo (Ebrahimian et al., 2010). This study aimed to clarify whether the PML protein is expressed and PML NBs are present in the earlier stages of sperm formation in the mouse testis. Our results indicate that the postnatal developing spermatogonial stem cells express PML proteins before going through mitosis and generation of mature sperm.

## Results

### PML expression in the outermost layer of seminiferous tubules

Our immunohistochemical findings showed that PML protein is expressed in the outermost cells of the seminiferous tubule, where developing spermatogonial stem cells are located (Fig. 1). This pattern of expression was observed at different time points after birth and during the maturation of the testis tissue. Immunocytochemistry on McCoy B fibroblasts confirmed the functionality of the antibodies, while immunohistochemical analysis of liver tissue was used as a positive control experiment for the detection of PML expression using fluorescence imaging.

### Oct4 and PML expression in the germinal epithelium of seminiferous tubules

Since PML protein seemed to be expressed in the postnatal developing spermatogonial stem cells, we analyzed the expression of OCT4 to evaluate the co-expression of these two proteins.

Immunohistochemical analysis of two mirror sections and H&E staining of the third serial section revealed that OCT4 and PML proteins are co-expressed in developing spermatogonial stem cells during the postnatal development of the testis (Fig. 2). The functionality of OCT4 and PML antibodies were tested in P19 and STO cell lines, respectively.

## Discussion

In the present study, we investigated the expression pattern of PML protein in the seminiferous tubules of the mouse testis during different stages of development after birth. In our previous study, the PML NBs were invisible in the mature sperm cells. However, the PML NBs were detectable after fertilization in the 2-cell stage embryo (Ebrahimian et al., 2010). Here we asked whether the PML protein is expressed and PML NBs are present in the earlier stages of sperm formation in the mouse testis. We found that the expression of PML protein is limited to the outermost cell layer of the seminiferous tubules from neonatal into mature life (Fig. 1). In the mirror sections of the testis, we noticed that PML protein is co-expressed with OCT4 (Fig. 2). Considering the anatomical location of these cells and since it is known that the OCT4 protein is expressed in and essential for the self-renewal of spermatogonial stem cells (Dann et al., 2008), we can conclude that the expression of PML protein is limited to the population of developing spermatogonial stem cells.

The role of PML protein in the self-renewal of leukemia cancer stem cells has been demonstrated (Wang et al., 1998; Welch et al., 2011). However, other studies have revealed that PML is involved in the maintenance of the hematopoietic and embryonic stem cells in physiological conditions (Chuang et al., 2011; Ito et al., 2008, 2012). The PML protein is involved in chromatin remodeling of the Oct4 gene promoter (Chuang et al., 2011). The nuclear receptor Tr2, as an activator for Oct4, is localized to and probably activated in the PML NBs (Gupta et al., 2008). The function of PML is not limited to the regulation of Oct4, and other stem cell maintenance factors including REST, Nanog, Stat3, and Myc interact with PML (Park et al., 2007). Developmental studies have also provided valuable evidence for the effect of PML on the fate of stem cells. For example, during the development of the mouse nervous system, the neural progenitor cells located at the neocortex express PML, and with the deletion of PML, the number of neural progenitor cells increases, causing the impairment of the cortex wall (Regad et al., 2009). In another study, knock out of the PML gene resulted in the disruption of normal mammary secretory epithelium development (Li et al., 2009).

Spermatogonial stem cells are a rare population of cells that are surrounded by a complicated environment. These cells have several molecular markers including PLZF, OCT4, SOX3, and NOTCH-1 (Hofmann et al., 2005; Meng et al., 2000; Pesce et al., 1998; Raverot et al., 2005; Von Schönfeldt et al., 2004; Yoshida et al., 2004). It is well established that OCT4 is a key transcription factor in the embryonic stem cells and together with SOX2 and NANOG creates the balance of self-renewal versus differentiation (Botquin et al., 1998; Boyer et al., 2005; Chambers et al., 2003; Mitsui et al., 2003; Niwa et al., 2000). It has been shown that the key stemness genes such as Nanog and Sox2 are associated with PML (Salmina et al., 2010; Tang et al., 2016). Our findings show that the PML protein is present in developing spermatogonial stem cells of developing mouse testis, even before earning the ability to produce mature sperms (Fig. 3). Thus, this could be an evidence for its role in the stemness and gene regulatory network during postnatal development of the spermatogonial stem cells.

## Conclusions

In conclusion, we have shown that PML protein is co-expressed with OCT4 in the outermost cellular layer of seminiferous tubules, where the spermatogonial stem cells are located during the development of mouse testis. The spermatogonial stem cells originate from the primordial germ cells (PGCs) and gonocytes which are tightly controlled by OCT4. Therefore, the next question would be whether PML is present in PGCs. Further studies will be needed to reveal the function of PML in the stemness of these cells.

## Methods

### Tissue samples

Adult male CD1 mice (weighing 200 g–250 g; 6 weeks of age) were purchased from the animal facility of Mashhad University of Medical Sciences and housed in stainless steel cages. The experiment was performed according to the guidelines for the Animal Care Committee of Ferdowsi University of Mashhad, Mashhad, Iran. Mice were kept in a controlled environment at  $25 \pm 3$  °C, the humidity of 40–65%, 12 h light/dark cycle, and free access to food and drinking water. The testes from one-day, two-, four-, and six-weeks old and adult (> 6 weeks of age) male mice were surgically removed and immediately placed in the fixator solution.

### Cell lines

McCoy B mouse fibroblasts, mouse fibroblast STO, and P19 mouse embryonal carcinoma cell lines were cultured in DMEM (Dulbecco's Modified Eagle Medium; Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) supplemented with 10% FBS (fetal bovine serum; Invitrogen GmbH, Darmstadt, Germany) and 10,000 units penicillin and 10 mg streptomycin/ml (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). All three cell lines were maintained at 37 °C in an atmosphere of 5% CO<sub>2</sub> in the air.

### Immunocytochemistry

All three cell lines were cultured on sterile coverslips in the culture medium and subjected to the immunocytochemical analysis. All stages of fixation, permeabilization, and immunolabelling were performed on cells cultured on coverslips. Cells were washed in phosphate-buffered saline (PBS) and were fixed in fresh 2% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, USA) in PBS (pH, 7.50) for 10 minutes at room temperature (RT). Fixed cells were washed three times with 1x PBS, permeabilized by 0.1% Triton X-100 in PBS for 5 min, and rinsed three more times with 1x PBS. Permeabilized cells were labeled with the mouse anti-promyelocytic leukemia protein monoclonal antibody (1:300; Chemicon/Millipore, Temecula, USA) for 2 hours at RT. After three washes with PBS, the donkey anti-mouse IgG labeled with Cy3 (1:500, Jackson ImmunoResearch Laboratories, Inc. West Grove,

USA) used as the secondary antibody. After three washes with PBS the immunolabelled cells were mounted on the slide in drops of anti-fade (0.1M propyl gallate dissolved in 90% glycerol in PBS) plus 300 nM 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St Louis, USA). Slides were stored in 4 °C and dark conditions before fluorescence imaging. Cells were imaged with a AH3-RFC fluorescence microscope (Olympus, Japan) equipped with D Plan Apochromat 20X UV and D Plan Apochromat 40X UV objectives and a DP71 digital camera (Olympus, Japan).

For Immunocytochemistry based on horseradish peroxidase (HRP), mouse fibroblast STO and P19 mouse embryonal carcinoma cell lines were quickly washed with 1x PBS and were fixed in fresh 2% paraformaldehyde in PBS (pH, 7.50) for 10 minutes at RT. Fixed cells were washed three times with 1x PBS and permeabilized with 0.5% Triton X-100 in PBS for 5 min. After washing with 1x PBS, 3% H<sub>2</sub>O<sub>2</sub> was added to remove the activity of endogenous peroxidases. After another washing with 1x PBS, cells were labeled with the mouse anti-promyelocytic leukemia protein monoclonal antibody (1:300; Chemicon/Millipore, Temecula, USA) for overnight at 4 °C. After another wash with PBS, the goat anti-mouse IgG-HRP secondary antibody (1:500) was added to the cells. After a wash with 1x PBS, diaminobenzidine (DAB) as a peroxidase substrate was added. Cells were washed with 1x PBS and rinsed three more times with pure ethanol and Xylen for 5 min. Finally, the cell samples were mounted using Entellan (Sigma-Aldrich, St Louis, USA) and were imaged under a BX51 light microscope (Olympus, Japan) equipped with UPlanSApo Plan Apochromat 20X and Plan C 40X/0.65na objectives, and a digital camera (Olympus, Japan).

## Histological preparations and immunohistochemistry

Testes from neonatal (postnatal day 1), juvenile (day 14 and 4 weeks old) and adult (> 6 weeks of age) mice were dissected and fixed in 4% PFA in PBS for 2 hours (for neonatal testes), or overnight (for juvenile and adult testes) at RT. Fixed tissues were processed for paraffin embedding. For each testis tissue, three 4 µm serial sections were cut from paraffin-embedded blocks. Sections were deparaffinized in xylene (two times, 15 min each), rehydrated in graded ethanol series (2 × 100% for 10 min, 1 × 95% for 5 min, 1 × 80% for 5 min, 1 × 70% for 5 min, 1 × 50% for 5 min) and rinsed in PBS (2 × 10 min). Then slides were incubated in the sodium citrate antigen retrieval buffer (10 mM Sodium Citrate, pH, 6.00) for 20 min at 97.5 °C and allowed to cool at room temperature. After rinsing twice in water and 1x PBS, non-specific binding sites were blocked by incubation with the blocking buffer (1 × PBS containing 1% bovine serum albumin) for 1 h at RT. Primary antibodies including mouse anti-PML and mouse anti-OCT4 (1:300) were diluted in the blocking buffer and added to tissue sections for an overnight incubation at 4 °C in a humid chamber. After two times washing in 1x PBS for 5 min, the sections were treated with 0.3% H<sub>2</sub>O<sub>2</sub> for 15 min at RT to block the endogenous peroxidase activity. Both desired proteins, PML and OCT4, from each mouse were incubated with the secondary antibodies (goat IgG or donkey IgG) conjugated with HRP (1:500) for 30 min at room temperature. Sections were washed two times with PBS for 5 min and treated with DAB. Also, one section from each mouse was stained by hematoxylin and eosin (H&E) which was used to evaluate testicular morphology. The slides were sealed in the mounting medium and studied

under a BX51 light microscope (Olympus, Japan) equipped with UPlanSApo Plan Apochromat 20X and Plan C 40X/0.65na objectives, and a digital camera (Olympus, Japan).

For Immunofluorescence staining, the dissected testes were embedded immediately in the OCT medium (Miles Scientific, Elkhart, USA) in the cryostat chamber (-30 °C). This system provided cut sections with 5 µm thickness which mounted on the poly-L-lysine (Sigma-Aldrich, St Louis, USA) coated slides. Before staining, the slides were warmed at RT for 30 minutes and fixed in 4% PFA for 20 minutes. Then, they were air-dried for 30 minutes. After washing with 1x PBS (2 × 10 min), permeabilization was performed in 0.1% Triton X-100 in PBS at RT for 10 min, which was followed by two rinses of 1x PBS. Nonspecific binding sites in tissue sections were blocked by incubation with the blocking buffer for 1 h at RT. The mouse anti-PML (1:300) primary antibody was diluted in the blocking buffer and added to tissue sections for overnight incubation at 4 °C in a humid chamber. After incubation, the slides were washed two times (10 min each) in 1x PBS. The secondary antibody labeling was performed using the donkey anti-mouse IgG-labelled cy3 (1:500, Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA) for 30 min at RT. Then, slides were washed two times (10 min each) in PBS. Drops of anti-fade plus DAPI was added to the immunolabelled slides. The edges of the coverslips were sealed with the nail polish and allowed to air-dry. Slides were kept in the cold (4 °C) and dark conditions before fluorescence imaging. Imaging was performed using an AH3-RFC fluorescence microscope (Olympus, Japan) equipped with D Plan Apochromat 20X UV and D Plan Apochromat 40X UV objectives and a DP71 digital camera (Olympus, Japan). Photomicrographs were analyzed using DP controller software.

## Abbreviations

PML: promyelocytic leukemia

PML NBs: Promyelocytic leukemia nuclear bodies

PGCs: primordial germ cells

SUMO: Small Ubiquitin-like Modifier

DAXX: Death domain-associated protein

Oct-4: octamer-binding transcription factor 4

SOX2: SRY-Box Transcription Factor 2

DAPI: 4',6-diamidino-2-phenylindole

PBS: Phosphate-buffered saline

DMEM: Dulbecco's Modified Eagle Medium

FBS: fetal bovine serum

HRP: horseradish peroxidase

DAB: 3,3'-Diaminobenzidine

H&E: hematoxylin and eosin

RT: room temperature

## **Declarations**

### **Ethics approval and consent to participate**

The ethical approval for this study was issued by the Committee on Research Ethics of the Ferdowsi University of Mashhad, based on the Ethical Guidelines of Research from The Ministry of Science, Research and Technology of Iran, and following the Declaration of Helsinki.

### **Consent for publication**

No identifying patient information is included in this report.

### **Availability of data and material**

The data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### **Competing interests**

The authors declare that they have no conflict of interest.

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This study was financially supported by Ferdowsi University of Mashhad, Iran.

### **Authors' contributions**

Conceived and designed the experiments: HD and AT. Performed the experiments: AT. Analyzed the data: AT and HD. Supervised the experiments: HD. Wrote the manuscript: AT and HD. All authors read and approved the final manuscript.



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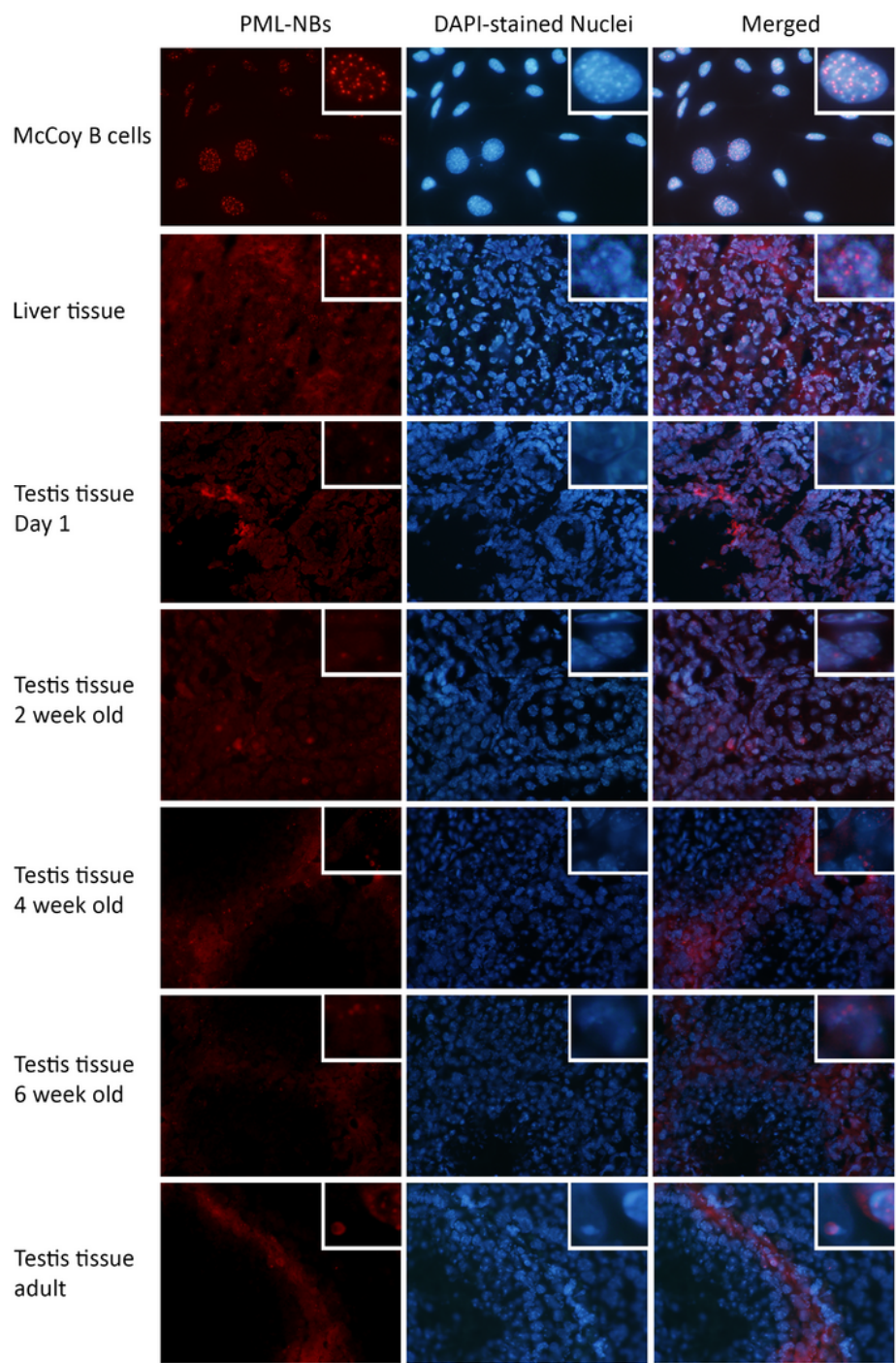
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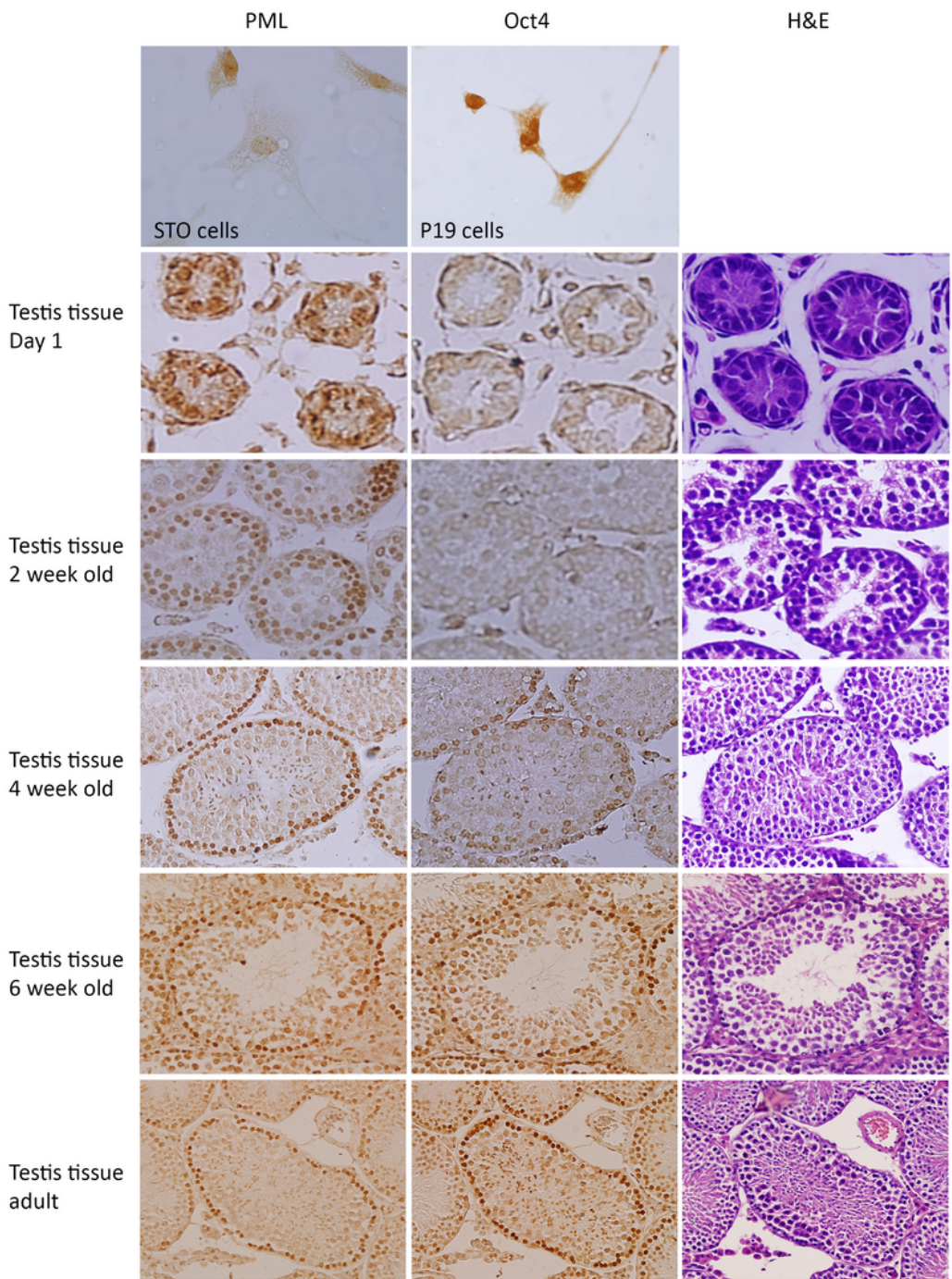
## Figures



**Figure 1**

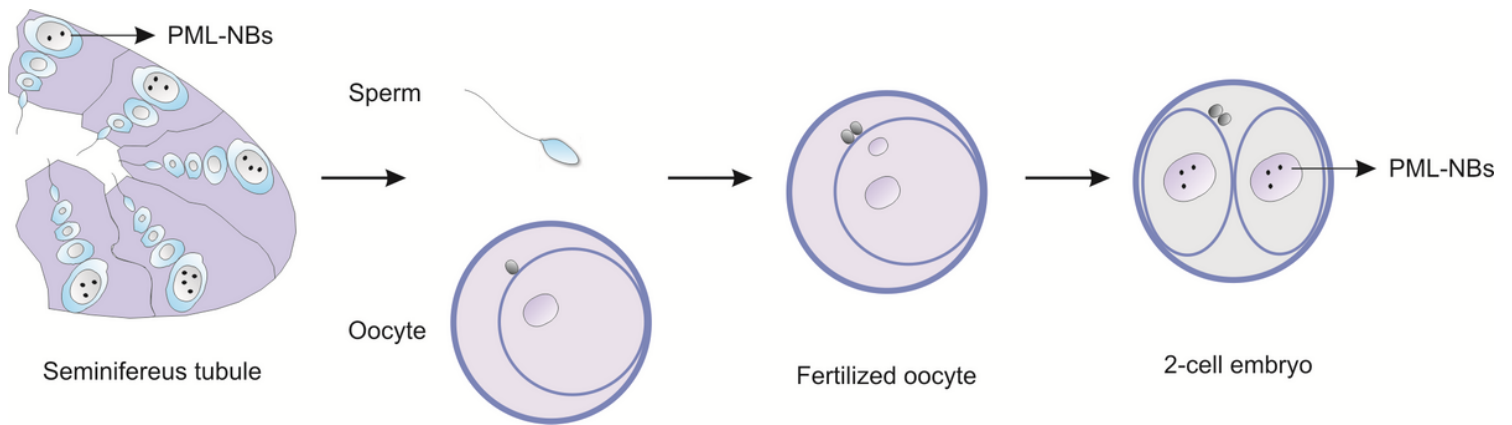
PML expression in the outermost layer of seminiferous tubules at different time points after birth and during maturation. The expression of PML protein was evaluated by immunocytochemistry in the nucleus of the McCoy B cell as a positive control. PML expression was identified as red immunofluorescence in the outermost layer of seminiferous tubules during development. For positive controls and each developmental stage, DAPI-stained nuclei and merged images are shown.





**Figure 2**

Expression of PML and OCT4 in the germinal epithelium of seminiferous tubules at different time points after birth and during maturation. The expression of OCT4 and PML proteins were detected in positive control P19 and STO cell lines, respectively. Mirror sections of mouse postnatal development were stained by H&E and immunohistochemistry for PML and OCT4 to identify the position of seminiferous tubular cells and to observe the co-expression of these signals in the testicular tissue.



**Figure 3**

An infographic depiction of PML expression and formation of PML NBs during mouse spermatogenesis, and early embryonic development. This image summarizes our findings in a previous study (Ebrahimian et al., 2010) and this study showing that the developing spermatogonial stem cells in seminiferous tubules contain PML NBs, while mature sperm and oocyte do not show PML NBs, even after fertilization in the 1-cell-stage embryo. The first appearance of PML NBs is in the 2-cell-stage embryo.