Supporting Online Material for

**Functional Hierarchy and Reversibility Within the Murine Spermatogenic Stem Cell Compartment**

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**This PDF file includes:**

Materials and Methods
SOM Text
Figs. S1 to S7
References

**Additional supplemental material includes the following:**

Movies S1 to S4
Supporting Online Materials

Materials and Methods

Animals: Transgenic mice used in this study, Ngn3/EGFP (S1), Ngn3/CreERTM (S2), and CAG-CAT-EGFP (S3), were maintained on the C57BL/6 background and mated to obtain individuals with the desired combination of transgenes. Artificial cryptorchid testes were prepared 2–3 months following the operation performed as described previously (S4). All the animals were maintained, bred, operated on, and sacrificed in the Animal Facility of Graduate School of Medicine and in accordance with the Animal Experiment Guidelines of Kyoto University.

Whole-mount immunofluorescence of seminiferous tubules: Untangled seminiferous tubules were fixed with 4% paraformaldehyde, 0.5 mM CaCl2, and phosphate-buffered saline (PBS) on ice for 4 h. The samples were attached to MAS-coated slide glass (Matsunami, Osaka, Japan) by half-drying. After incubation in 0.2% NP40 in PBS for 20 min, the samples were dehydrated through a methanol series (25, 50, 75, and 100% in PBS containing 0.1% Tween 20 [PBS-T]) on ice, followed by rehydration in PBS-T. After blocking with 1% bovine serum saline (BSA) and 4% donkey serum (Jackson ImmunoResearch, PA, USA) in PBS-T for 1 h, specimens were incubated in 1% BSA, 4% donkey serum and 0.5 mM CaCl2 in PBS-T containing the appropriate combination of the following antibodies at 4°C overnight: polyclonal anti-GFP antibody (1:500; MBL, Nagoya, Japan, catalog #598), polyclonal anti-GFRα1 antibody (2 µg/ml; R&D systems, MN, USA, catalog #AF560), monoclonal anti-GFP antibody (a gift from A. Imura), monoclonal anti-E-cadherin antibody (2 µg/ml; Takara Bio, Shiga, Japan, clone #ECCD-2), monoclonal anti-PLZF antibody (1:50; Calbiochem, OR, USA, clone #2A9), and polyclonal anti-MVH antibody (a gift from T. Noce (S5)). After washing with PBS-T, an appropriate second antibody (Alexa Fluor 488- or Alexa Fluor 594-conjugated donkey anti-rabbit, anti-rat, anti-mouse, or anti-goat IgG (1:500, Molecular Probes, CA, USA)) was applied to the samples at room temperature for 2 h. After washing with PBS-T, specimens were mounted in Permafluor (Beckman Coulter, CA, USA) for observation under a DMRBE upright fluorescence microscope (Leica, Wetzlar, Germany) equipped with an Axiocam CCD camera (Carl Zeiss Vision, Oberkochen, Germany).
Analyses of cyst length and gene expression of spermatogonia: To determine the cyst length, after immunofluorescence staining with anti-E-CAD antibody, the whole mount seminiferous tubule specimens were observed under a fluorescence microscope. The E-CAD staining coupled with staining for TEX14, a protein that specifically localizes to intercellular bridges (S6), enabled us to reliably identify syncytial cysts of spermatogonia and adjacently located separate clones based on the E-CAD staining, in the absence of co-stained TEX14 signal. In many experiments double-staining was done with anti-E-CAD and a second antibody to determine the gene expression as well.

Throughout, Ngn3-expressing cells were identified using Ngn3/EGFP transgenic mouse testes by means of detecting EGFP protein. Therefore, these are formally to be designated as Ngn3-expressing cells or Ngn3/EGFP-expressing cells. In this study, however, they are designated as NGN3+ cells to avoid confusion with the cells that were pulse-labeled with constitutive expression of EGFP induced by 4OH-tamoxifen in Ngn3/CreERTM;CAG-CAT-EGFP double transgenic mice.

Pulse-label of the Ngn3+ spermatogonia: Three-month-old Ngn3/CreERTM;CAG-CAT-EGFP double transgenic male mice were injected intraperitoneally with 2 mg 4OH-tamoxifen (Sigma, MO, USA) dissolved in ethanol, dimethyl sulfoxide, and sesame oil (Nakalai Tesque, Kyoto, Japan). After incubation with or without regeneration induced by busulfan administration (see below), their testes were processed for whole-mount immunofluorescence.

Induction of regeneration: Ngn3/EGFP transgenic adult mice were injected intraperitoneally with busulfan (10 mg/kg, Wako Pure Chemical Industries, Osaka, Japan). According to the experimental schedule, their testes were processed for whole-mount immunofluorescence. The frequencies of the stained cells were counted over extended segments of seminiferous tubules measuring at least 25 mm in length for each specimen under a DMRBE upright fluorescence microscope (Leica).

For the pulse-regeneration experiments, three-month-old Ngn3/CreERTM;CAG-CAT-EGFP transgenic mice were administered tamoxifen 2 d before busulfan injection. The testes were removed according to the experimental schedule and processed for immunostaining.
Segments of seminiferous tubules measuring at least 240 mm were analyzed for each specimen.

**Live imaging of Ngn3-expressing spermatogonia:** Live imaging of the GFP-expressing spermatogonia in Ngn3/EGFP mice was preformed as described previously (S7). Live-imaging during regeneration (i.e., several days following busulfan treatment) has not been achieved currently because the residual systemic effect of busulfan makes the anesthesia difficult to control.

**Supplemental Text**

**Text S1: E-CAD and PLZF mark the entire Aₐ, Aₚ₀, and Aₐ₁ population**

Expression of E-CAD and PLZF is mostly overlapped in Aₐ, Aₚ₀ and Aₐ₁ spermatogonia (Fig. S2A), whose morphology (i.e., the cyst length) is best determined after stained with E-CAD. As shown in Fig. S2C, the frequencies of E-CAD⁺ Aₐ, Aₚ₀, and Aₐ₁ relative to Sertoli cell nuclei were very similar to those determined by standard criteria, i.e., nuclear morphology and inter-nucleus distance in hematoxylin-stained whole mount specimens (S8, 9). In addition, as shown in Fig. S2D, in the experimental cryptorchid testes where Aₐ, Aₚ₀, and Aₐ₁ are the only surviving germ cells (S4), E-CAD stained the entire germ-cell population defined by the expression of MVH (S10). These results strongly suggest that E-CAD/PLZF represents the entire population of Aₐ, Aₚ₀, and Aₐ₁, and that the possible contribution of E-CAD/PLZF double negative Aₐ, Aₚ₀, and Aₐ₁ would not be substantial (S11-13).

**Text S2: Estimation of the labeling efficiency of NGN3⁺ spermatogonia**

Labeling efficiencies after pulse-labeling of Ngn3/CreER™, CAG-CAT-EGFP mice (percentage of the GFP⁺ spermatogonial cysts) were calculated for Aₐ, Aₚ₀, and Aₐ₁ fractions, using the frequencies of the labeled cells and the Ngn3/GFP-positive spermatogonia (Fig. S3A). The true labeling efficiency (percentage of the labeled cells) in Aₐ is identical to the calculated value (33%). However, estimations of the true labeling efficiencies of Aₚ₀ and Aₐ₁ are complicated and
include several assumptions: EGFP protein induced in one or more cells within the syncytium would be distributed through the intercellular bridges to spread all over the cyst, which could also dilute the GFP signal below the detection threshold. Therefore, we evaluated the hypothesis that the NGN3+ A₆, A₇, and A₈ are labeled at random with the same probability as that of A₆ (33%). Based on this assumption, the probability (P) that k cells are labeled within an n-cell cyst follows a binomial distribution, indicated by the following equation:

\[ P = \frac{n!}{k!(n-k)!} p^k (1-p)^{n-k} \quad \text{for} \quad k = 0, 1, 2, \ldots, n \quad (\text{eq. 1}) \]

where p is the probability for labeling a cell (0.33, in this case). Based on this equation, the probability of labeling an n-cell cyst (n = 1, 2, 4, 8, 16) with detectable GFP intensity above the presumptive threshold (t) is provided as a summation of P for all the k where k/n ≥ t. As shown in Fig. S3B, the observed percentage of labeled cysts (red) satisfactorily fits the theoretically expected profiles when the threshold is set at 1 labeled cell out of 3.5 cells. We theorize that this threshold is reasonable. Therefore, it follows that labeling was induced in ~30% of NGN3+ cells regardless of the number of chained cells.

Captions Supplemental Figures

Fig. S1: Classification of spermatogonial populations in the mature mouse testis.

(A) The spermatogenic cells observed in the mature mouse testis are classified primarily based on their ploidy and cell cycle status (mitosis or meiosis). Spermatogonia are defined as diploid germ cells that undergo mitosis.

(B) The spermatogonia are further classified. First, based on the nuclear morphology, they are classified into ‘type-A’ which do not exhibit apparent chromatin condensation, and ‘type-B’ which show prominent heterochromatin condensation. ‘Intermediate’ type (In) are also classified.

In the advanced portion of ‘type-A’ spermatogonia of relatively longer cysts (A₁ through A₄), cell cycle progresses in a synchronized manner among cysts that are closely located. As a result, A₁→A₂→A₃→A₄ transitions occur synchronously (each accompanying one cell cycle) in continuous segments of the seminiferous tubules, in accordance with the seminiferous epithelial cycle (S1/4). This is followed by synchronous transition into In, B spermatogonia, and then into
spermatocytes (each step accompanying one cell cycle). This is why spermatogonia from A₁ through B are often termed ‘differentiating spermatogonia’ collectively.

In contrast, a minor and primitive population of ‘type-A spermatogonia’, do not show synchronous cell cycle progression. They are single cells or short cysts, and further classified based on their cyst length into Aₐ, Aₜₐ, Aₐ₁₄, Aₐ₁₆, and Aₐ₃₂ (single cells and 2, 4, 8 or 16 cell cysts, respectively), as described in the main text. Aₐ₃₂ spermatogonia are also generated occasionally. In literatures, Aₐ, Aₜ and Aₐ₃₂ spermatogonia have been often collectively designated as ‘undifferentiated spermatogonia’ or ‘undifferentiated type-A spermatogonia (Aₐ₃₂)’.

**Fig. S2: E-CAD and PLZF mark the entire Aₛ, Aₜₛ, and Aₐ population**

(A) Whole-mount immunofluorescence of seminiferous tubules for E-CAD (magenta) and PLZF (green), with the summarized counts below. Scale bar, 100 µm.

(B) Frequency of E-CAD⁺ cysts per cyst size. Note that most cysts include 2ⁿ cells.

(C) Comparison of the frequency of the E-CAD⁺ spermatogonia (this study) and that of Aₛ, Aₜₛ, and Aₐ spermatogonia identified based on the authentic morphological criteria, per 1000 sertoli cells (S₉).

(D) E-CAD expression in Aₛ, Aₜₛ and Aₐ spermatogonia in cryptorchid testes. Whole-mount immunofluorescence of seminiferous tubules from cryptorchid testes, which harbor Aₛ, Aₜₛ and Aₐ spermatogonia, but lack A₁ and more advanced spermatogonia, spermatocytes and spermatids (S₄). All germ cells detected by MVH expression (green) (S₅) were also positive for E-CAD (magenta). Positive cell counts are shown under the panels. Note that all of the residual germ cells that survived in cryptorchid testes are E-CAD⁺. Small cells with weak staining for the anti-MVH antibody were residual Sertoli cells. Scale bar, 100 µm.

**Fig. S3: Labeling efficiency of NGN3⁺ spermatogonia by 4OH-tamoxifen**

(A) Calculation of the labeling efficiency (i.e., the percentage of the labeled syncytium), 2 d after administration of 4OH-tamoxifen to Ngn3/CreERT²;CAG-CAT-Z mice. Labeling efficiency was estimated based on the frequency of NGN3⁺ cysts (A) and frequency of the pulse-labeled cysts (shown as cyst number) (B). These frequencies were determined as relative occurrences compared to E-CAD⁺ cysts using whole-mount double immunofluorescence for
E-CAD and GFP on seminiferous tubules from Ngn3/GFP transgenic mice and the induced transgenic mice described above.

(B) Expected probabilities of labeled cysts that harbor GFP above the threshold of detection (1 labeled cell out of 16, 8, 4, 3.5, 3, and 2, as indicated) were calculated as described in the following discussion. The observed percentage of labeled cysts (“observed,” shown in red) fits well with the prediction for the detection threshold of 1 out of 3.5 (blue). See Suppl. Text S2 for details.

Fig. S4: Derivation of the vast majority of KIT$^+$ spermatogonia from NGN3$^+$, but not from GFRα1$^+$, population

(A) Double immunofluorescence of Ngn3/EGFP mouse seminiferous tubules for GFP (green) and KIT (magenta). The green fluorescence was intensified so that weak, residual GFP signals (used here as a short-term lineage tracing marker) could be visualized. Upper panels are the stages observed immediately after KIT expression was initiated in young differentiating spermatogonia (likely A$_1$ or A$_2$), while lower panels show the area harboring more advanced spermatogonia, which are small in size and denser, with KIT expression being stronger than that in the upper panel. Note that eventually all the KIT$^+$ differentiating spermatogonia are also positive for residual GFP signals, indicating that they are derived from NGN3$^+$ cells.

(B) Immunofluorescence of adult mouse seminiferous tubule stained for GFRα1 and KIT. Arrowheads and arrowheads indicate GFRα1 single-positive and double-positive cells, respectively. Frequencies of single/double-positive cells are shown under panels. Note that only a small fraction of KIT$^+$ spermatogonia are also GFRα1$^+$. Assuming that GFRα1, a cell surface protein, behaves as a short-term tracer, suggests that only a few GFRα1$^+$ spermatogonia transform into KIT$^+$ spermatogonia. Scale bars, 100 µm.

Figure S5: Summary of cellular events observed during live-imaging of Ngn3/EGFP mouse testes

Summation of gain of Ngn3/EGFP signal (A), doubling divisions (B) and clone fragmentation (C) observed in the live-imaging of Ngn3/EGFP transgenic mouse testes. Total area of 3.5 mm$^2$ (which equals to the area of 29 visual fields under a 20x objective lens) was filmed from 8 mice for approximately 3-4 days. (A) Gain of Ngn3/EGFP expression categorized as shown.
Divisions of GFP\(^{+}\) A\(_{4}\) that give rise to two single spermatogonia and that which double the cyst length. non-2\(^{n}\), cysts including cells of different numbers from 2\(^{n}\). uncertain, cysts whose cell numbers could not be determined precisely. (C) Schematic presentation of all the three cysts that exhibited fragmentation during the abovementioned record periods, while several possible but uncertain cases were also observed. Movies of cysts #1, #2 and #3 are shown in Mov. S2, S3, and S4, respectively. Cyst #1 is also represented in Fig. 4. The numerals indicate the number of cells within a cyst, while those in parentheses (A\(_{4}\)a-16 in cyst #1 and A\(_{4}\)a-8 in cyst #2) appeared for such short periods that it was not possible to discriminate which occurs first between cell division or cyst fragmentation (Mov. S2 and S3), suggesting a close relationship between these events. Also note that cyst fragmentation does not occur in a stereotypic way, but a variety of fragmentation patterns are observed in such a small number of cases.

**Fig. S6: Absolute number of the GFP-tagged GFRα1+ A\(_{4}\) cells observed in steady-state spermatogenesis and regeneration**

(A) Experimental schedule. Ngn3/CreERT\(^{TM}\); CAG-CAT-EGFP adult mice were administered with 4OH-tamoxifen. 2, 10, and 20 days later, testes were analyzed for the GFP-tagged GFRα1\(^{+}\) A\(_{4}\) spermatogonia, which is the same series of experiments shown in Fig. 3. To induce regeneration, marginal dose of busulfan (10mg/kg body weight) was injected on day 2, followed by the same series of analyses, which was the same that show in Fig. 5. 3 months later, persistent GFP\(^{+}\) patches were counted in hemeostasis and regeneration (S15).

(B) Summary of the estimated absolute number of GFP-tagged GFR α 1\(^{+}\) A\(_{4}\) per testis without or with busulfan injection, respectively, on indicated days. The same data for Fig. 3A-B and 5F-I were used. In steady-state spermatogenesis, estimations were based on the count of observed GFP-tagged GFRα1\(^{+}\) A\(_{4}\), the length of the observed segments, and the total seminiferous tubules length per testis that was measured in our previous report (S15). In regeneration, number of Sertoli cells was also counted in the shrinking seminiferous tubules, and the total cell number were estimated based on the assumption that the total Sertoli cell number is constant between undisturbed and regenerating testes. In addition, number of the discrete GFP\(^{+}\) patches observed after three months patches are shown. Results are shown as average ± SEM per testis. *P=0.04, **P=0.03, ***P<0.001 (Student \(t\)-test).

(C) Fluorescent stereomicrographs of the untangled seminiferous tubules from the entire testes 3 months after pulse. Note a larger number of GFP\(^{+}\) patches (arrows) in busulfan-treated testes.
than in uninjected one, compatible to results shown in (S15).

**Fig. S7: Emergence of GFR α 1⁺ Aₘ₈ and Aₘ₁₆ during regeneration.**

(A) Percentage of GFR α 1⁺ Aₘ₈ (circles) and Aₘ₁₆ (squares) out of total (E-CAD⁺) Aₘ₈ and Aₘ₁₆, respectively, detected at different times after busulfan administration. Values (average ± SEM) were calculated from counts of three testes for each time point. Percentages were compared with that of 0 d. *P=0.002 and **P<0.001 (χ² test).

(B) A GFR α 1⁺ Aₘ₁₆ cyst observed in seminiferous tubule of a regenerating testis 18 d after busulfan administration. Inset indicates the position of the cells. This class of cysts was barely observed in steady-state spermatogenesis. Scale bar, 100 µm.

**Captions for Supplemental Movies**

**Movie S1: Gain of Ngn3/EGFP expression in Ngn3-negative Aₙ, Aₚ and Aₘ₈ spermatogonia**
Live-imaging of a Ngn3/EGFP mouse testis showing the gain of Ngn3/EGFP signal in Aₙ, Aₘ₄ and probable Aₘ₈. The selected frames of this movie are represented in Fig. 2I. The elapsed time is shown in days:hours:mins., as is also the case in all of the movies below.

**Movie S2: Fragmentation of a syncytial cyst of Aₘ₈**
Live-imaging of a Ngn3/EGFP mouse testis representing cyst#1, which underwent fragmentation as described in Fig. 4 and Fig. S5C.

**Movie S3: Fragmentation of a syncytial cyst of Aₘ₄**
Live-imaging of a Ngn3/EGFP mouse testis representing cyst#2, which underwent fragmentation as described in Fig. S5C. Right panel tracks the cells with dots. Cells shown by the same color are derived from the same single cell within Aₘ₄ at the beginning of the record. Note that the fragmented cells rapidly lost the Ngn3/EGFP signal, which made it difficult to trace the complete lineage. Also note that this recorded field has overlap with a movie that was
published previously (S7).

**Movie S4: Fragmentation of a syncytial cyst of Aal-4**
Live-imaging of a Ngn3/EGFP mouse testis representing cyst#3, which underwent fragmentation as described in Fig. S5C.

**References for Supporting Online Materials**

Nakagawa et al., Suppl. Fig. S1

A

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Nakagawa et al., Suppl. Fig. S2

A

PLZF⁺/E-CAD⁺ = 970 (99.7%) PLZF⁺/E-CAD⁻ = 3 (0.3%) PLZF⁻/E-CAD⁺ = 0 (0%)

B

C

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D

MVH⁺/E-CAD⁺ = 233 (100%) MVH⁺/E-CAD⁻ = 0 (0%) MVH⁻/E-CAD⁺ = 0(0%)
Nakagawa et al., Suppl. Fig. S3

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B

![Graph showing clones over detection threshold](chart.png)

- observed
- theoretical
- ≥ 1/16
- ≥ 1/8
- ≥ 1/4
- ≥ 1/3.5
- ≥ 1/3
- ≥ 1/2
Nakagawa et al., Suppl. Fig. S4

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GFRα1⁺/KIT⁻ = 609 (98.2%)  GFRα1⁺/KIT⁺ = 11 (1.8%)
Nakagawa et al., Suppl. Fig. S5

A. gain of Ngn3 expression

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B. doubling division of Ngn3\(^+\) spermatogonia

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C. fragmentation of Ngn3\(^+\) cysts

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<td></td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>elapsed time (hours)</th>
<th>cyst #3</th>
<th>0</th>
<th>20</th>
<th>40</th>
<th>60</th>
<th>80</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

※ loss of Ngn3/EGFP signal
A Ngn3/CreER\textsuperscript{TM};CAG-CAT-EGFP mice

\textit{labeling} busulfan

\begin{itemize}
  \item \textbf{day 0}
  \item \textbf{day 2}
  \item \textbf{day 10}
  \item \textbf{day 20}
  \item \textbf{3 months}
\end{itemize}

\begin{itemize}
  \item count GFP-tagged GFRα\textsuperscript{1+} A
  \item count GFP+ patches
\end{itemize}

B

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Bus. injection & analysis point & No. GFP-tagged GFRα\textsuperscript{1+} A per testis & No. patches per testis \\
\hline
- & 2d & 377.2 ± 61.3 & \\
- & 10d & 67.9 ± 35.6 & \\
- & 20d & 29.7 ± 15.0 & \\
- & 3m & 3.8 ± 0.5 & * \\
\hline
+ & 2d & 377.2 ± 61.3 & \\
+ & 10d & 104.2 ± 12.9 & \\
+ & 20d & 119.2 ± 43.1 & **
\hline
+ & 3m & 11.8 ± 1.9 & ***
\hline
\end{tabular}
\end{table}

C

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{busulfan_unini}
\end{figure}
Nakagawa et al., Suppl. Fig. S7

A

%GFRα1+ cysts

0 3 5 8 10 18
days after busulfan

B

GFRα1

Inset: Imaging of GFRα1 expression.