

Mouse Spermatogenic Stem Cells Continually Interconvert between Equipotent Singly Isolated and Syncytial States

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SUMMARY

The identity and behavior of mouse spermatogenic stem cells have been a long-standing focus of interest. In the prevailing “ A_s model,” stem cell function is restricted to singly isolated (A_s) spermatogonia. By examining single-cell dynamics of $GFR\alpha 1+$ stem cells *in vivo*, we evaluate an alternative hypothesis that, through fragmentation, syncytial spermatogonia also contribute to stem cell function in homeostasis. We use live imaging and pulse labeling to quantitatively determine the fates of individual $GFR\alpha 1+$ cells and find that, during steady-state spermatogenesis, the entire $GFR\alpha 1+$ population comprises a single stem cell pool, in which cells continually interconvert between A_s and syncytial states. A minimal biophysical model, relying only on the rates of incomplete cell division and syncytial fragmentation, precisely predicts the stochastic fates of $GFR\alpha 1+$ cells during steady state and post-insult regeneration. Thus, our results define an alternative and dynamic model for spermatogenic stem cell function in the mouse testis.

INTRODUCTION

In mammalian testes, spermatogenic stem cells are responsible for both the continual production of sperm in steady state and regeneration following injury (de Rooij and Russell, 2000; Meis-

trich and Van Beek, 1993; Yoshida, 2012). However, the dynamics of the stem cell population remain largely unresolved at the single-cell level. The process of spermatogenesis takes place in seminiferous tubules (Figure 1A). All stages of germ cells are nourished by somatic Sertoli cells, which support a prominent network of tight junctions that separate the basal and adluminal compartments and, together with the basement membrane, provide the structural basis of the tubules. The tubules are surrounded by peritubular cells, whereas the intertubular space is made up of a network of blood vessels and interstitial cell types. Spermatogonia (mitotic germ cells that include stem cells) lie in close association with the basement membrane in the basal compartment. When meiosis begins, cells detach from the basement membrane and translocate across the tight junctions, after which they undergo meiotic divisions and spermiogenesis, and mature sperm are released into the lumen. This organization is observed uniformly throughout the entire 1.7 m tubule length that constitutes a single mouse testis (Russell et al., 1990), suggesting that seminiferous tubules lack a discrete anatomically defined niche.

In mouse, spermatogonia are divided into “undifferentiated” and “differentiating” populations (Figures 1A and 1B). “Undifferentiated spermatogonia” are found as singly isolated cells (A_s) or syncytia consisting mainly of 2 (A_{pr}), 4 (A_{al-4}), 8 (A_{al-8}), or 16 (A_{al-16}) cells. The formation of syncytia is due to “incomplete division,” a germline-specific cell division process by which cytokinesis does not complete and cytoplasmic connection between daughter cells persists via intercellular bridges (de Rooij and Russell, 2000; Russell et al., 1990). This process continues through subsequent mitotic and meiotic divisions, resulting in the extension of syncytia from differentiating spermatogonia (A_1 , A_2 , A_3 , A_4 , Intermediate, and B) to haploid spermatids.

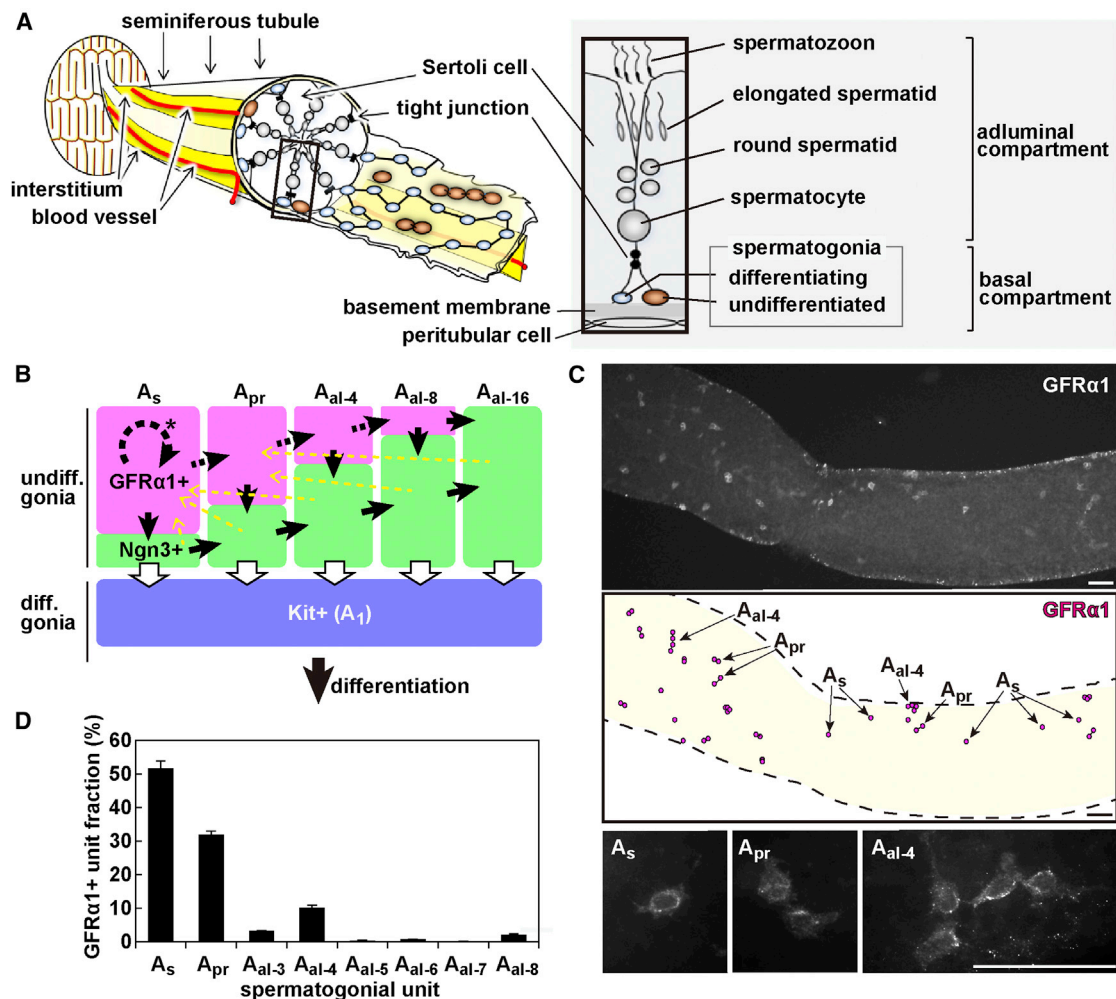


Figure 1. GFR α 1+ Spermatogonia in Mouse Seminiferous Tubules

(A) Anatomy of seminiferous tubules. Undifferentiated spermatogonia (brown) and differentiating spermatogonia (blue) are distributed among Sertoli cells in the basal compartment (see text for details).

(B) A proposed hierarchy of GFR α 1+ and Ngn3+ subpopulations of undifferentiated spermatogonia, as well as Kit+ differentiating spermatogonia (modified from Nakagawa et al., 2010). Black and white solid arrows indicate processes that have been directly observed, whereas the black broken arrows represent presumptive dynamics of GFR α 1+ cells, in which only GFR α 1+ A_s self-renew (asterisk). Yellow broken arrows indicate the processes of “reversion,” which occur infrequently in steady state.

(C) Immunofluorescence for GFR α 1 in whole-mount seminiferous tubule specimen. Middle panel: distribution of GFR α 1+ spermatogonia. Lower panels: higher magnification of GFR α 1+ A_s, A_{pr}, and A_{al-4}. Scale bars, 50 μ m.

(D) Composition of GFR α 1+ spermatogonial units observed in adult mouse testis. Averages \pm SEM from three testes are shown.

Experimentally, posttransplantation colony-formation and regeneration assays established that, whereas Kit-positive (Kit+) differentiating spermatogonia seem to retain some stem cell potential, the vast majority of stem cell function is restricted to Kit-negative (Kit-) undifferentiated spermatogonia (Barroca et al., 2009; Ohbo et al., 2003; Shinohara et al., 2000). Based on the detailed analyses of fixed specimens, it was proposed in 1971 that stem cell activity may be restricted to the population of A_s spermatogonia, whereas interconnected A_{pr} and A_{al} syncytia are irreversibly committed to differentiation and no longer contribute to the stem cell pool (Huckins, 1971; Oakberg, 1971). This hypothesis, which has become the prevailing theory, is known as the “A_s model.”

The population of undifferentiated spermatogonia is further divided according to their heterogeneous gene expression (Hofmann et al., 2005; Nakagawa et al., 2010; Sada et al., 2009; Suzuki et al., 2009a; Yoshida et al., 2004, Yoshida, 2012; Zheng et al., 2009). In undisturbed steady-state spermatogenesis, the GFR α 1+ subpopulation (mainly A_s, A_{pr}, and fewer A_{al}) is thought to reside on the top of the hierarchy (Nakagawa et al., 2010; Sada et al., 2009). As well as maintaining their own population, GFR α 1+ cells also give rise to the second, Ngn3+, subpopulation of undifferentiated spermatogonia (comprised of more A_{al} and fewer A_s and A_{pr}) (Figure 1B). Previous studies using live imaging and cre-mediated pulse labeling of Ngn3+ spermatogonia have demonstrated that the majority of

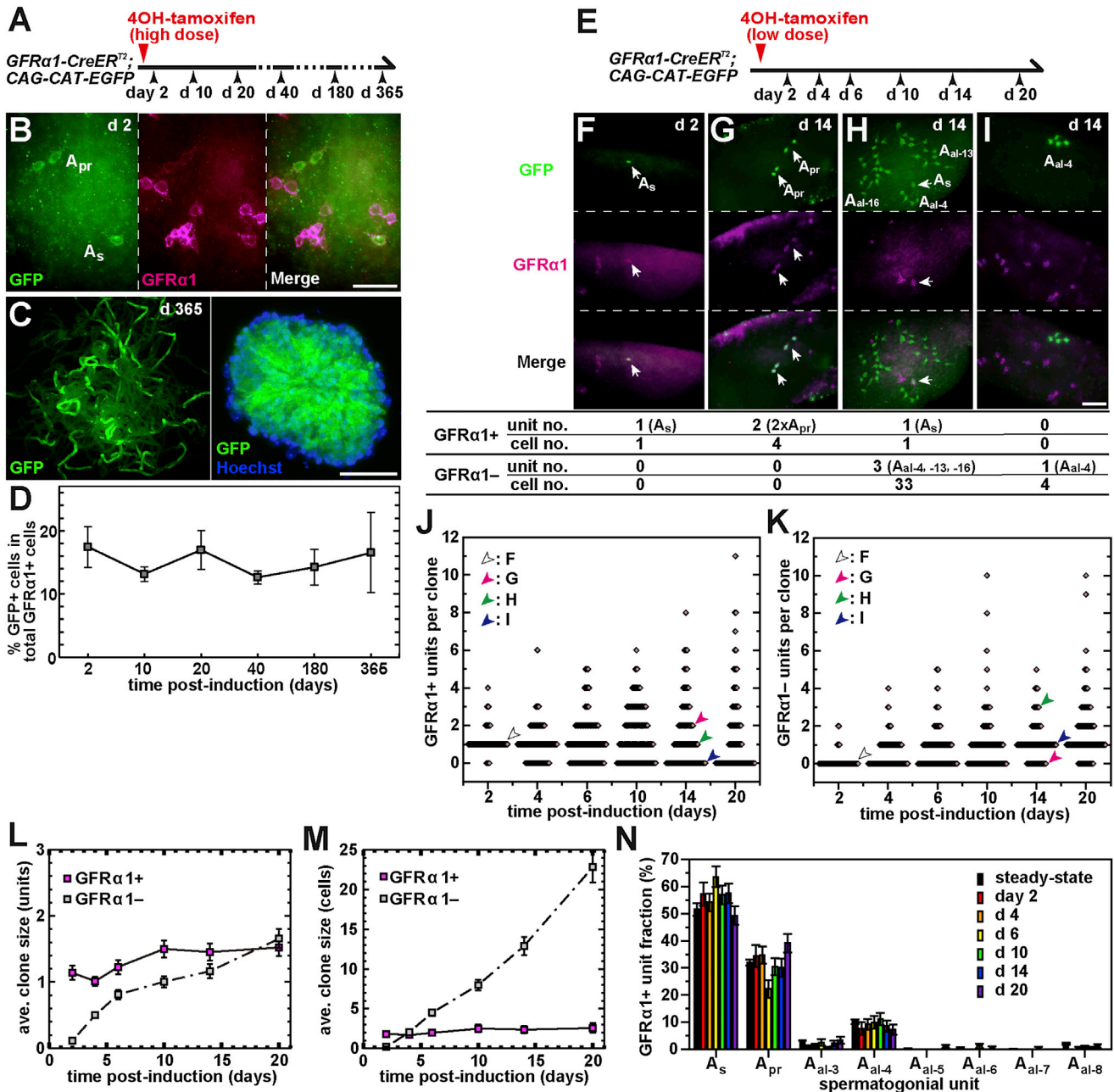


Figure 2. Pulse-labeling Analyses of *GFRα1+* Spermatogonia

(A) Experimental schedule for (B)–(D). *GFRα1-CreER^{T2}; CAG-CAT-EGFP* mice were administered with 2.0 mg 4OH-tamoxifen to pulse label *GFRα1+* units with GFP, and their testes were analyzed at the indicated time points.

(B) Labeling of a fraction of *GFRα1+* cells (magenta) with GFP expression (green) 2 days after pulse.

(C) Untangled seminiferous tubules at 365 days postlabeling, showing numerous patches of GFP+ cells (left) and a cross-section of such a patch in which GFP signal is found in all stages of germ cell differentiation (right).

(D) Fraction of GFP+ cells out of total *GFRα1+* population from 2 to 365 days postinduction. Averages ± SEM from 3, 4, 4, 3, 3, and 3 testes for 2, 10, 20, 40, 180, and 365 days postinduction are shown, respectively.

(E) Experimental schedule for clonal fate analysis of pulse-labeled *GFRα1+* units in (F)–(N). *GFRα1-CreER^{T2}; CAG-CAT-EGFP* mice were administered with 0.35 mg 4OH-tamoxifen to sparsely label the *GFRα1+* spermatogonia at an efficiency of 1.0% ± 0.1% (n = 3) and analyzed at the indicated time points.

(F–I) Whole-mount staining of seminiferous tubule for GFP (green) and *GFRα1* (magenta) at 2 (F) and 14 (G, H, and I) days postinduction; stains are scored as shown below. Arrows indicate the labeled *GFRα1+* units.

(J and K) Distribution of clone size as measured by *GFRα1+* (J) and *GFRα1-* (K) unit number per clone over time. Each dot indicates one clone. The clones shown in (F)–(I) are plotted as shown by white, magenta, green, and blue arrowheads, respectively.

(legend continued on next page)

Ngn3+ cells transfer to the next Kit+ differentiating spermatogonia (Nakagawa et al., 2007, 2010). Intriguingly, these studies also revealed that Ngn3+ cells retain the capability of regaining GFR α 1 expression, fragmenting into single cells or shorter syncytia (through breaking of intercellular bridges), and contributing to the long-term stem cell pool (Figure 1B, yellow dotted arrows), suggesting that the entirety of undifferentiated spermatogonia (both GFR α 1+ and Ngn3+) may contribute to stem cell activity. However, such “reversion” of Ngn3+ cells takes place only infrequently in steady state but becomes prevalent in regeneration following tissue insult or transplantation. Therefore, Ngn3+ cells have been considered as a reserve population, whereas GFR α 1+ cells are thought to represent the primary population responsible for the stem cell function (Nakagawa et al., 2007, 2010; Spradling and Fan, 2010).

Such flexible behavior of Ngn3+ cells, especially syncytial fragmentation, questions the premise of the “A_s model” that syncytia are irreversibly committed to differentiation. It was also shown that, in common with other Ngn3+ spermatogonia, Ngn3+ A_s cells are strongly biased toward differentiation to Kit+, indicating that not all the A_s spermatogonia function equally as stem cells (Nakagawa et al., 2010). In addition, based on the occasional contribution of Ngn3+ cells to the long-term stem cell pool, the behavior of the pulse-labeled stem cells was analyzed for months to over a year. The results demonstrated that stem cells are continually and stochastically lost and replaced by their neighbors, through a process of population asymmetry (Klein et al., 2010; Nakagawa et al., 2007). This observation not only further challenged the “A_s model” but also questioned the cellular basis of stem cell loss and replacement.

To summarize, the preceding analyses of how Ngn3+ spermatogonia behave over time (by means of pulse labeling and live imaging) have questioned the validity of the “A_s model,” at least in its original form. Although A_s spermatogonia that are GFR α 1+ were presumed to be the primary stem cell type (Nakagawa et al., 2010) (Figure 1B, black dotted arrow with asterisk), this conjecture lacks direct experimental support. To understand the stem cell dynamics, therefore, it is essential to dissect the fate behavior of GFR α 1+ spermatogonia over time. In this study, we developed knockin mouse models and conducted intravital in vivo live-imaging and pulse-labeling studies of GFR α 1+ spermatogonia at a single-cell resolution. On the basis of the unexpected behavior of GFR α 1+ cells observed in these studies, we propose an alternative theory of the identity and behavior of mouse spermatogenic stem cells.

RESULTS

Stem Cell Function of GFR α 1+ Spermatogonia

GFR α 1+ spermatogonia lie scattered unevenly on the basement membrane of seminiferous tubules (Figures 1A and 1C). Intriguingly, however, their local density over a prolonged tubule length

(several millimeters) was found to be remarkably constant in adult mice, with 17 ± 1 “units” of GFR α 1+ spermatogonia per mm of tubule. Here, we define “a unit” as either “an A_s spermatogonium” or “a single syncytium consisting of multiple spermatogonia.” Their composition was also conserved between individuals, not only for A_s, A_{pr}, and A_{al-4}, which comprised some 50%, 30%, and 10% of total units, respectively, but also for the small numbers of A_{al-8} and “odd-sized” syncytia (e.g., 3-, 5-, and 6-cell chains designated hereafter as A_{al-3}, A_{al-5}, and A_{al-6}), which together constitute the remaining 10% (Figure 1D).

To trace the fate of GFR α 1+ spermatogonia, we developed a knockin mouse model that enables the pulse labeling of GFR α 1+ cells with persistent GFP expression, without disturbing the tissue architecture, following a single administration of 4OH-tamoxifen to GFR α 1-CreER^{T2};CAG-CAT-EGFP mice (Figures 2A and 2B; Figures S1A and S1B available online). After partial (~20%) pulse labeling of this population, long-term chase (months to over a year) revealed that labeled cells formed contiguous patches in which GFP+ cells were found in all stages of differentiation (Figure 2C). Importantly, the percentage of GFP+ cells out of the total GFR α 1+ spermatogonia remained constant over the same extended period (Figure 2D). This indicates that the initially labeled GFR α 1+ population (comprised of around 6,000 units = some 20% of the 30,000 total GFR α 1+ units per testis) continually reproduced approximately the same number of GFR α 1+ spermatogonia and gave rise to differentiating descendants that lost GFR α 1 expression. This finding also shows that the influx of cells from outside the GFR α 1+ compartment (i.e., Ngn3+, Kit+, or other unknown cells) is minimal in this undisturbed condition, consistent with previous reports (Nakagawa et al., 2007, 2010). To conclude, in steady state, the population of GFR α 1+ spermatogonia function as the stem cells.

Intricate Clonal Fate Behavior of Pulse-Labeled Individual GFR α 1+ Spermatogonia

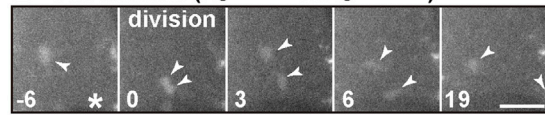
Then we were motivated to investigate how individual GFR α 1+ spermatogonia behave to achieve such population-level stem cell dynamics, using the same mouse model. With a lower dose of 4OH-tamoxifen, label was introduced into GFR α 1+ cells sparsely (one labeled unit per ~6 mm tubule length on average), so that the fate of each “clone” (defined as a cohort of cells derived from a single labeled unit, regardless of whether or not they have split into multiple units) can be analyzed (Figure 2E). Over the time course of 2–20 days postinduction, the constituent units of individual clones were scored according to their GFR α 1 expression and unit length by whole-mount immunostaining of seminiferous tubules. Shortly (2 days) after induction, the majority of labeled clones contained a single GFR α 1+ unit (Figures 2F–2K). However, intriguingly, the subsequent clonal fates did not follow a regular pattern, but were highly variable between clones: for example, at day 14, some clones were observed to

(L and M) Average number of GFR α 1+ and GFR α 1– units (L) and cells (M) over the total clones. In (M), syncytia of 32 or more cells, all of which were GFR α 1– and observed 4 or more days after the pulse, were scored as 32-cell syncytia because of the difficulty in making a precise count; this method underestimates the number of GFR α 1– cells (broken line).

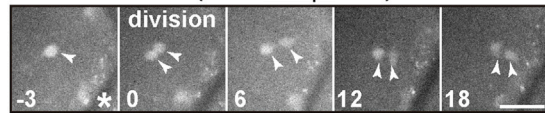
(N) Composition of the unit length of total pulse-labeled GFR α 1+ spermatogonia over time, compared with steady-state tissue composition. Data in (L)–(N) show averages \pm SEM ($n = 3, 4, 5, 4, 4, 6,$ and 3 testes for $2, 4, 6, 10, 14,$ and 20 days postinduction, respectively). Scale bars, $50 \mu\text{m}$ throughout. The row data for (J)–(N) are shown in Table S1.

A	unit	total observed time (total unit number)	events	products	counts	ave. rate (event/day)
A _s		7982 h (171)	cell division	2xA _s A _{pr}	2 33	1/166 1/10
			death		7	1/48
			cell division	A _{al-4}	10	1/12
A _{pr}		2779 h (70)	fragmentation	2xA _s	5	1/23
			death		0	—
			n.d.			
A _{al-3}		63 h (3)	n.d.			
A _{al-4}		1287 h (31)	cell division	A _{al-8}	4	1/13
			fragmentation	4xA _s	1	1/7
				2xA _s +A _{pr}	3	
				A _s +A _{al-3}	3	
			death		1	0
A _{al-8}		72 h (4)	n.d.			

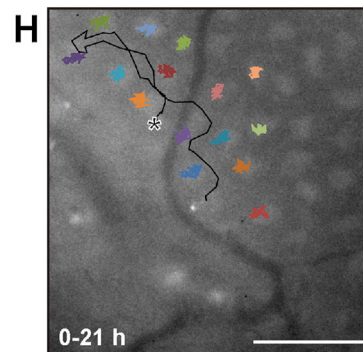
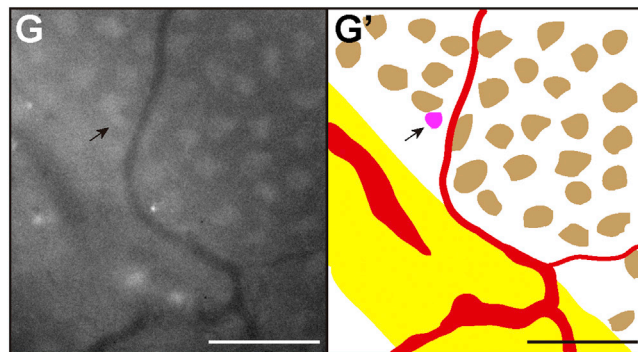
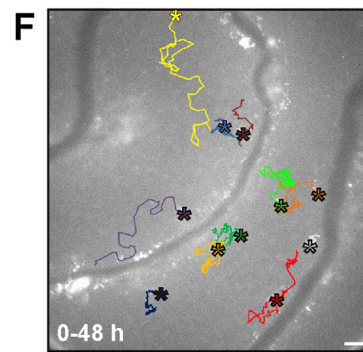
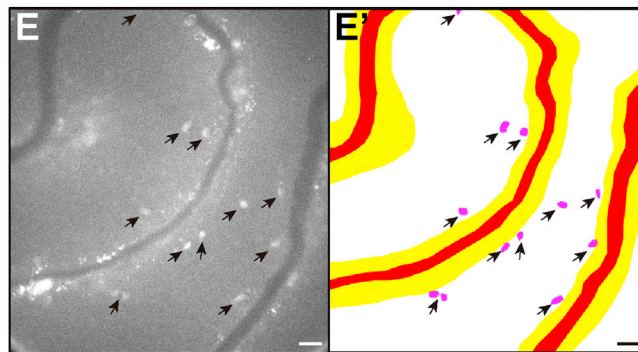
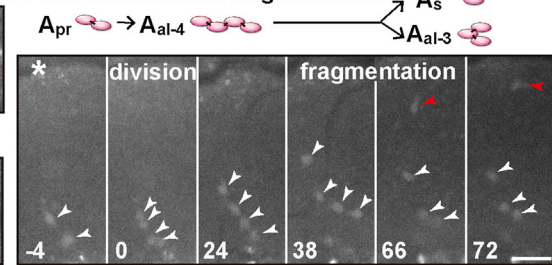
B cell division (A_s → 2xA_s)



C cell division (A_s → A_{pr})



D cell division and fragmentation



contain multiple $GFR\alpha1+$ units only (where the $GFR\alpha1+$ unit number increased without producing differentiation-destined $GFR\alpha1-$ progeny) (Figure 2G); some contained one $GFR\alpha1+$ and multiple $GFR\alpha1-$ units (where the $GFR\alpha1+$ unit number was maintained, while producing $GFR\alpha1-$ progeny) (Figure 2H); and others contained $GFR\alpha1-$ units only (where the $GFR\alpha1+$ unit was lost altogether) (Figure 2I). The degree of clonal variation in the number of $GFR\alpha1+$ and $GFR\alpha1-$ units (and cells) broadened progressively over time (Figures 2J, 2K, and S2; Table S1).

Despite the apparent variability in the individual clonal fates, the average of more than 100 clones at each time point recovered a conventional steady-state stem cell behavior through this period: in particular, the average number of $GFR\alpha1+$ units per clone remained close to one, whereas the number of $GFR\alpha1-$ units (and, more dramatically, $GFR\alpha1-$ cells) per clone steadily increased (Figures 2L and 2M). Further, the composition of $GFR\alpha1+$ units with different lengths across numerous clones remained largely constant over time, commensurate with that of the total $GFR\alpha1+$ population observed by immunostaining of fixed samples (Figure 2N). Altogether, these findings are consistent with the dynamics of population asymmetry, in which maintenance of stem cells and production of differentiating descendants are balanced at the population level through continuous loss and replacement of stem cells (Klein and Simons, 2011).

Cell Division and Syncytial Fragmentation of $GFR\alpha1+$ Spermatogonia Observed by Live Imaging

We then investigated the behavior of $GFR\alpha1+$ spermatogonia by means of *in vivo* live imaging of $GFR\alpha1-EGFP$ knockin mouse testis (Uesaka et al., 2007) (Figure S1C), exploiting a procedure reported previously (Yoshida et al., 2007). Theoretically, the observed increase in cell number per pulse-labeled clone indicates the process of cell division (either complete or incomplete), whereas the increase in unit number per clone provides evidence of complete division ($A_s \rightarrow 2 \times A_s$) and/or syncytial fragmentation. Indeed, all of these expected processes were observed directly in the live imaging. Since continuous live imaging was feasible up to ~ 3 days, the average rates of these processes were measured by collecting data from multiple time courses (Figure 3A).

Unexpectedly, following a total of $\sim 8,000$ hr of observation, only two cases out of 35 divisions of $GFR\alpha1+ A_s$ cells were found to be complete, leading to the generation of two A_s spermatogonia (translating to a rate of once per 5–6 months), whereas the vast majority of divisions were incomplete and gave rise to

one A_{pr} (Figures 3A–3C; Movies S1 and S2). Within a syncytium (A_{pr} and A_{al}), cell division was always incomplete and synchronous, leading to the doubling of syncytial length (e.g., $A_{pr} \rightarrow A_{al-4}$). Of particular note, fragmentations of $GFR\alpha1+$ syncytia were observed at a frequency much higher than that of $Ngn3+$ syncytia (estimated at around once per 4 months per bridge) (Nakagawa et al., 2010) and even comparable to that of cell division (Figures 3A and 3D; Movie S3). This effectively replenishes the shorter units lost through incomplete divisions. Because of the half-life of the EGFP protein (2–3 days), the live-imaging study could not resolve their transition to $Ngn3+$ cells, based on their loss of EGFP fluorescence during the filming time available. However, the clonal fate of pulse-labeled $GFR\alpha1+$ spermatogonia 2 days postinduction indicated that the $GFR\alpha1+ \rightarrow Ngn3+$ transition occurred in all categories of A_s , A_{pr} , and A_{al} $GFR\alpha1+$ spermatogonia (Figure S3A), consistent with previous live imaging of *Ngn3-EGFP* mouse testes (Nakagawa et al., 2010). The death of $GFR\alpha1+$ units was observed only rarely (Figure 3A).

Altogether, these observations indicate that $GFR\alpha1+$ cells continually change their states between A_s , A_{pr} , and A_{al} spermatogonia through a combination of incomplete division and syncytial fragmentation, while giving rise to $Ngn3+$ cells from all of these states.

Rates of Incomplete Division and Syncytial Fragmentation of $GFR\alpha1+$ Spermatogonia

In addition to these qualitative implications, the live-imaging study further provides quantitative insight into the dynamics of $GFR\alpha1+$ cells. First, the rate of cell division (essentially incomplete) appeared to be independent of unit length because A_s , A_{pr} , and A_{al-4} syncytia all divide at around once per 10 days (Figure 3A). Second, the average fragmentation frequency of A_{pr} (one bridge) was around once per 20 days, whereas that of A_{al-4} (three bridges) was proportionately higher at around once per 7 days (Figure 3A), suggesting that each bridge breaks around once per 20 days, independent of unit length. Intriguingly, the fragmentation of A_{al-4} syncytia provided, instead of a regular pattern, fragments involving all possible permutations, viz. $4 \times A_s$, $2 \times A_{pr}$, $2 \times A_s + A_{pr}$, or $A_s + A_{al-3}$ (Figure 3A), at frequencies compatible with stochastic breakdown of intercellular bridges once a syncytium is licensed for fragmentation (Figure S3B). Therefore, incomplete cell division and syncytial fragmentation of $GFR\alpha1+$ spermatogonia appear to occur at constant rates, independent of the unit length.

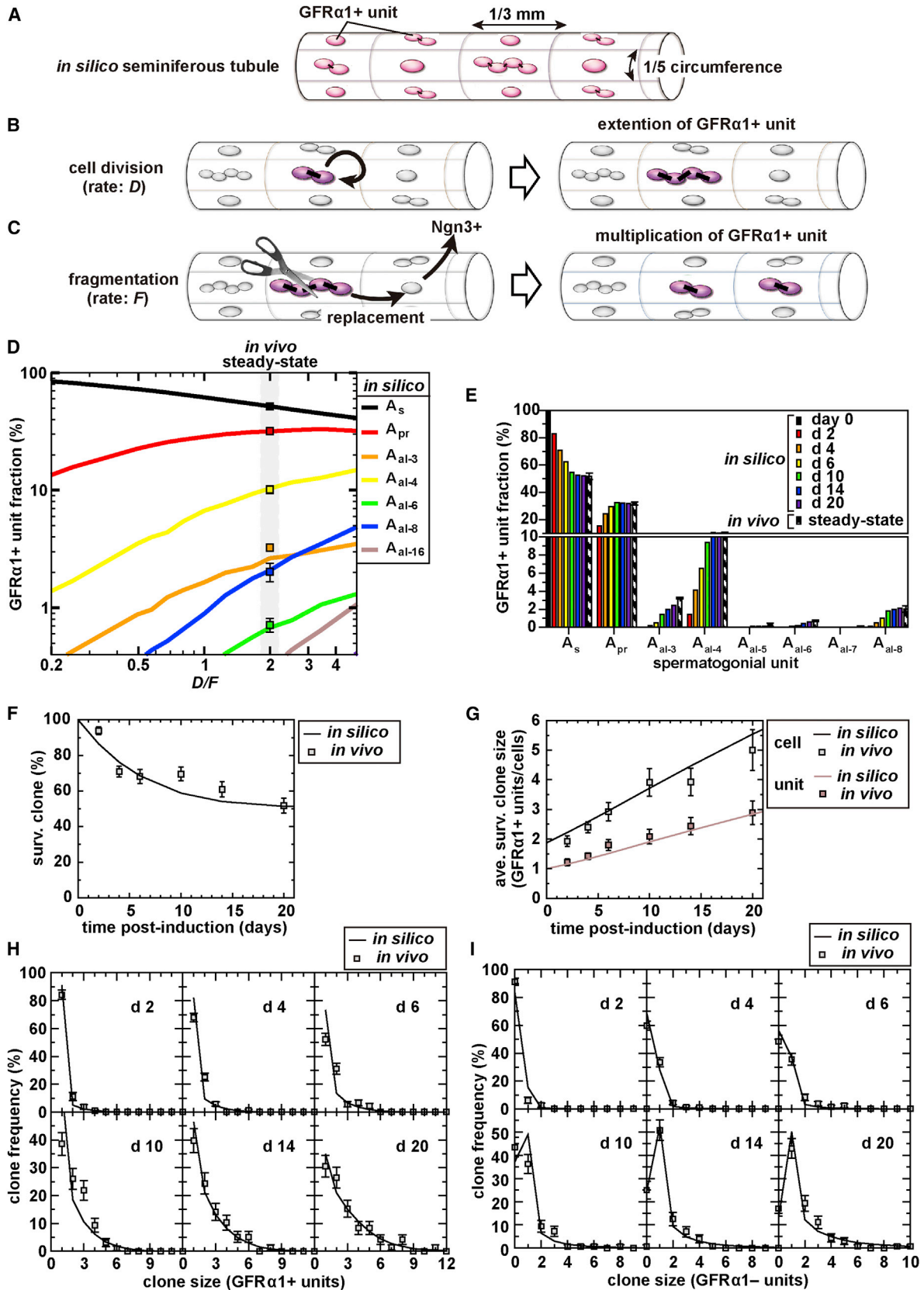
Figure 3. Dynamics of $GFR\alpha1+$ Spermatogonia Observed by Live Imaging

(A) Summary of cell division, fragmentation, and death of $GFR\alpha1+$ spermatogonia observed in live imaging of $GFR\alpha1-EGFP$ knockin mouse testes. Average rates of each event are calculated as “counts of observed events”/“total observation time.” nd, not detected.

(B–D) Examples of an $A_s \rightarrow 2 \times A_s$ division (B), an $A_s \rightarrow A_{pr}$ division (C), and an $A_{pr} \rightarrow A_{al-4}$ division, followed by a fragmentation into an A_s (red arrowhead) and an A_{al-3} (D), shown in selected frames of Movies S1, S2, and S3, respectively. Arrowheads, $GFR\alpha1-GFP+$ spermatogonia; numerals, elapsed time relative to the cell division (hr). Asterisks, blood vessels.

(E and F) Localization and movement of $GFR\alpha1+$ spermatogonia observed in live imaging. (E and E') A representative image of the surface of $GFR\alpha1-EGFP$ mouse testis (the first frame of Movie S4) is shown. (E') Trace of (E) showing $GFR\alpha1+$ spermatogonia (magenta), blood vessels (red), and interstitium (yellow). (F) Trajectories of individual $GFR\alpha1-GFP+$ spermatogonia over 48 hr of observation (Movie S4), shown in different colors.

(G and H) Movement of $GFR\alpha1+$ spermatogonia among the immobile Sertoli cells. (G) The first frame of the live imaging of $GFR\alpha1-EGFP$; $GATA1-EGFP$ mouse testis (Movie S5). (G') Trace of (G) showing a $GFR\alpha1+$ spermatogonium (magenta), Sertoli cells (brown), blood vessels (red), and interstitium (yellow). (H) Trajectories of a $GFR\alpha1-EGFP+$ spermatogonium (black line) and $GATA1-EGFP+$ Sertoli cells (colored lines) during 21 hr of observation, overlaid on the first frame. Asterisks indicate the starting positions; scale bars, 30 μm .



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Active Movement of GFR α 1+ Spermatogonia around the Vasculature-Associated Region

In common with the entire population of undifferentiated spermatogonia (Chiarini-Garcia et al., 2001; Yoshida et al., 2007), the GFR α 1+ subpopulation tends to localize near the vasculature and interstitium surrounding the tubules (Figures S3C, S3C', and S3F–S3G'). Moreover, from the live-imaging study, it was apparent that GFR α 1+ spermatogonia were in constant movement in the basal compartment (Figures 3E, 3E', and 3F; Movie S4). Whereas the majority preferentially moved within the vasculature-proximal region, others migrated from one such region to another. Without showing any systematic pattern, cells were seen to actively weave their way through the ordered network of immobile Sertoli cells (Figures 3G, 3G', and 3H; Movie S5), over a range of approximately 20–150 μ m within a single day. This contrasts with the behavior of Ngn3+ spermatogonia, which are less motile in the vasculature-associated region, before actively spreading over the basal compartment on transition into A₁ spermatogonia (Yoshida et al., 2007).

Synthesis of a Minimal Biophysical Modeling Scheme

Considering the aforementioned observations of highly variable clonal fates, continual conversion between the states of A_s, A_{pr}, and A_{ai} and active movement in the tissue, the behavior of GFR α 1+ spermatogonia may seem unconstrained. However, the observation that incomplete division and syncytial fragmentation occur at constant rates, independent of unit length, may suggest simple rules underlying such complex behaviors. We were motivated, therefore, to try to capture the dynamics of GFR α 1+ spermatogonia using a biophysical modeling scheme. In particular, we synthesized a model relying solely on parameters inferred from live imaging (the foregoing two rates), as well as their density and localization in seminiferous tubules. Then the validity of the model was evaluated by testing whether it was able to predict the wide range of independent data, including the intricate clonal fates revealed by the pulse-labeling study. Here, the model was designed to be as simple as possible, with minimal parameters, so that we could capture the basic principles of the dynamics of GFR α 1+ spermatogonia, as described below. For further details of the modeling scheme, see the [Supplemental Experimental Procedures](#).

As seen above, although the GFR α 1 units are scattered and moving around the tissue, their local density (viz. pool size) is maintained largely constant. In formulating the model, we aimed

to capture the dynamics of GFR α 1+ units under the condition of such a constant density. To reflect this, we considered a modeling scheme in which the basal compartment of the seminiferous tubules was divided into domains that accommodate one GFR α 1+ unit each (Figure 4A). Based on their measured average density (17 units/mm tubule) and affinity to the vasculature (Figures S3C, S3C', and S3F–S3G'), whose average number around a tubule is 5.2 (Klein et al., 2010), we divided the circumference into five domains of 1/3 mm in length (Figure 4A). In this scheme, each domain corresponds to the approximate territory of a single GFR α 1+ unit.

Then to reflect our live-imaging observations, all the constituent GFR α 1+ spermatogonial units were allowed stochastically to undergo incomplete cell division that doubles the unit length, at a constant rate (defined as D), independent of unit length (Figure 4B). In addition, intercellular bridges were allowed to break stochastically, leading to the fragmentation of a syncytium into multiple units, at a constant rate (defined as F) per bridge throughout all the GFR α 1+ syncytia (Figure 4C). The pattern of fragmentation was set to occur randomly among the bridges (with any bridge breaking with a probability of 50%), consistent with observation (Figure S3B). To achieve a constant density of GFR α 1+ units, the genesis of a new GFR α 1+ unit by fragmentation was set to accompany the GFR α 1+ \rightarrow Ngn3+ transition in any one of the neighboring domains and the replacement of the lost GFR α 1+ unit by the newly formed GFR α 1+ unit (Figure 4C). Such translocation between domains was consistent with the observed movements of GFR α 1+ cells (Figures 3E–3H).

To reduce the complexity of the model, we did not include the infrequent process of A_s \rightarrow 2 \times A_s complete division (Figures 3A and 3B), which is in any case implicit in the sequential occurrence of an A_s \rightarrow A_{pr} division, followed by A_{pr} \rightarrow 2 \times A_s fragmentation. Nor did we allow for the death of GFR α 1+ spermatogonia or Ngn3+ \rightarrow GFR α 1+ reversion, reflecting the low frequency of these processes (Figure 3A) (Nakagawa et al., 2010). Reflecting their low fragmentation frequency, we could assume that GFR α 1– (Ngn3+ and more advanced) units do not fragment but simply accumulate at each lattice site. Finally, GFR α 1– units were also set to follow a low rate of death (once per 30 days), consistent with observation (Huckins and Oakberg, 1978). However, within the framework of the model, death of GFR α 1– units does not, in any case, affect the dynamics of the GFR α 1+ population.

Figure 4. Model Prediction of the In Vivo Dynamics of GFR α 1+ Spermatogonia

(A) An imaginary seminiferous tubule used as the framework for the modeling scheme: the basal compartment is modeled as a regular cylindrical lattice, in which each domain accommodates one GFR α 1+ unit.

(B and C) Elementary processes introduced into the model. With the rate of " D ," a GFR α 1+ spermatogonial unit divides incompletely to double its length (B). With the rate of " F " per bridge, a GFR α 1+ syncytium fragments into multiple pieces; this event is allied with the GFR α 1+ \rightarrow Ngn3+ transition of neighboring unit(s). As a result, newly generated units replace neighboring units and persist as GFR α 1+ (C). For details, see the main text and the [Supplemental Experimental Procedures](#).

(D) Dependence of the steady-state unit composition on the ratio D/F predicted in silico (multicolored lines), in which the rates measured from live imaging ($D = \text{once}/10 \text{ days}$; $F = \text{once}/20 \text{ days/bridge}$; $D/F = 2.0$) captured the in vivo steady-state composition obtained from whole-mount immunostaining (squares).

(E) Convergence in silico to steady-state composition of GFR α 1+ units from an initial condition in which all GFR α 1+ units are A_s, using the rate constants $D = \text{once}/10 \text{ days}$ and $F = \text{once}/20 \text{ days/bridge}$.

(F–I) Model prediction captures clonal fate behaviors of GFR α 1+ units observed in vivo over the 20 day time course, represented by a percentage of surviving clones out of total clones (F), average number of GFR α 1+ units/cells in individual surviving clones (G), and clone size distribution for GFR α 1+ (H) and GFR α 1– (I) units. Throughout, lines show the in silico predictions using the same D and F rates, whereas the experimental data are shown by squares (average \pm SEM among testes). (H) and (I) are replotted from Figures 2J and 2K.

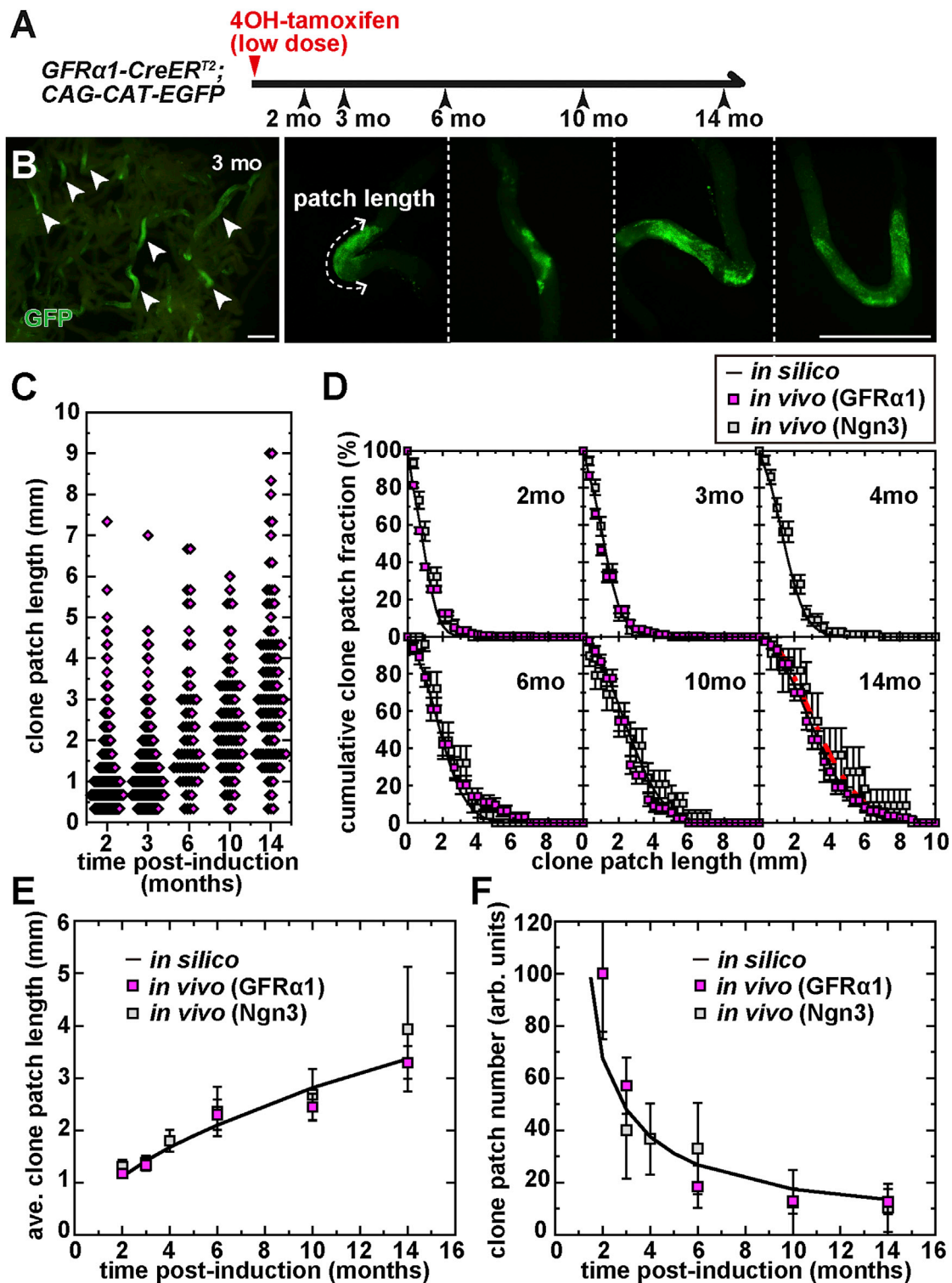


Figure 5. Long-Term Dynamics of $GFR\alpha1$ + Spermatogonia-Derived Clones

(A) Experimental schedule for the long-term clonal analysis of pulse-labeled $GFR\alpha1$ + cells.

(B) Seminiferous tubules at 3 months postlabeling, showing GFP+ clonal patches (arrowheads) and their higher magnifications with measurement of the patch length. Scale bars, 1 mm.

(C) Distribution of clonal patch lengths at 2, 3, 6, 10, and 14 months postinduction.

(legend continued on next page)

Model Prediction of the In Vivo Dynamics of GFR α 1+ Spermatogonia

We then questioned whether the model had the capacity to predict the wide and complex range of independent in vivo measurements. In this scheme, the in silico dynamics of GFR α 1+ spermatogonia is fully specified by just two parameters: the rates of cell division (D) and fragmentation (F). First, within the framework of the model, the GFR α 1+ population is predicted to converge to steady state, in which GFR α 1+ units acquire a particular composition that is independent of the initial condition but depends uniquely on the ratio D/F (colored lines in Figure 4D). Intriguingly, using the rates of D (= once per 10 days) and F (= once per 20 days per bridge) inferred from live imaging, the model faithfully recapitulated the steady-state composition of GFR α 1+ spermatogonia measured in vivo (Figure 4D). The steady state is recovered rapidly (largely within 10 days, corresponding to one round of cell division on average), even from such an extreme initial condition in which all GFR α 1+ units are A_s (Figure 4E). In addition to the proportions of GFR α 1+ A_s , A_{pr} , and A_{al-4} spermatogonia, the model correctly predicted the near-absence of GFR α 1+ units larger than eight, as well as the small number of “odd-sized” units (A_{al-3} , A_{al-5} , A_{al-6} , and A_{al-7}), which was already a nontrivial test of the validity of the modeling scheme.

Second, we evaluated the extent to which the model can predict the detailed clonal fate dynamics of GFR α 1+ units scored in the pulse-labeling experiment over 20 days postinduction (Figures 2E–2N; Figure S2; Table S1), with the same rates of D and F . In common with the in vivo observation (Figures 2J, 2K, and S2), in silico clones also followed variable fates. In the model, once the GFR α 1+ \rightarrow Ngn3+ transition occurs in all the units of a clone, such a clone never returns to the GFR α 1+ compartment. As a result, the fraction of clones that retained at least one GFR α 1+ unit (surviving rate) progressively diminishes over time (line in Figure 4F), which quantitatively recapitulated the in vivo measurements (squares in Figure 4F). As a consequence of the steady-state dynamics, it was also predicted that the average number of GFR α 1+ units (and cells) of the “surviving” clones progressively increase, so that the average number of GFR α 1+ units across all clones remains close to one. Indeed, these predictions quantitatively captured the in vivo observations (Figures 2L, 4G, and S4A). More significantly, the model prediction showed excellent agreement with the in vivo distribution of GFR α 1+ units (and cells) at all data points over the time course of 20 days (Figures 2J, 4H, S2, and S4B). Finally, the average and distribution of the number of GFR α 1– units per clone (i.e., units that had exited GFR α 1+ compartment and transited to Ngn3+ and then more advanced spermatogonia) were also accurately predicted by the model (Figures 2K, 2L, 4I, and S4A).

To summarize, from an in silico modeling scheme that was synthesized solely from the local density and distribution of GFR α 1+ units in seminiferous tubules and the rates of cell division and syncytial fragmentation inferred from the live-

imaging study, we were able to accurately predict both the steady-state tissue composition of spermatogonial units (an independent measurement) and the intricate fate behavior of spermatogonial units (obtained from a totally independent pulse-labeling study). These findings provide strong support for the validity of the simple modeling scheme in capturing the in vivo steady-state behavior of GFR α 1+ population, at least over the 20 day time course.

Long-Term Dynamics of GFR α 1+ Population

We then assessed the capacity of the same model to predict the long-term (over months) clonal behavior in steady state. On this timescale, clones derived from the pulse-labeled single GFR α 1+ units evolved into large (sometimes fragmented) patches in which the labeled GFR α 1+ cells were overwhelmed in number by differentiating labeled cells. Because a count of unit(cell) number is unfeasible in this phase, we characterized clone size by the patch length along the axis of the tubule (Figures 5A and 5B). In common with the number of GFR α 1+ units(cells) in each clone seen in the short-term pulse-labeling study up to 20 days, at 2, 3, 6, 10, and 14 months postinduction, the patch length showed a variable size distribution, with the average length increasing over time (Figures 5C–5E). In parallel, the number of surviving patches per testis decreased (Figure 5F). Significantly, extrapolation of the in silico dynamics to these longer times correctly predicted the average and distribution of patch length and surviving rate of GFR α 1+ unit-derived clones, using the same rates of cell division and fragmentation, D (= once per 10 days) and F (= once per 20 days per bridge) (Figures 5D–5F).

In previous studies, pulse labeling of Ngn3+ spermatogonia was used to trace the fate of surviving stem cell clones over a 14 month time course (Klein et al., 2010; Nakagawa et al., 2007). Because the majority of Ngn3+ cells are destined for differentiation, these studies relied on the premise that the few Ngn3+ spermatogonia that had transited back into the GFR α 1+ compartment behaved without distinction from the “innate” GFR α 1+ cells (Nakagawa et al., 2010). This premise is strongly supported by the observation that the average and distribution of patch lengths as well as the rate of surviving clones are all consistent between GFR α 1+ and Ngn3+ cell-derived clones and predicted by the same model at these long times (Figures 5D–5F).

To summarize, the minimal biophysical model, which was synthesized from the very short-term observations in live imaging (up to 3 days), is capable of capturing the fate behavior of GFR α 1+ spermatogonia from the short term (up to 20 days) to the long term (up to times comparable with the life span of mice).

Dynamics of GFR α 1+ Spermatogonia Following Tissue Insult

We then turned to investigate the dynamics of GFR α 1+ spermatogonia following a strong perturbation from steady state.

(D) Comparison of clonal patch length distribution between in silico prediction (solid lines) and in vivo measurement (squares) over 14 months. Red dotted line in the panel of 14 months shows the scaling function obtained by Klein et al., 2010.

(E and F) Comparisons of the evolution of average patch length (E) and patch number per testis presented in arbitrary units (F) between in silico prediction (solid line) and in vivo measurements (squares). In (D), (E), and (F), magenta and gray squares indicate patches originated from GFR α 1+ (replotted from C) and Ngn3+ units (replotted from Klein et al., 2010 and Nakagawa et al., 2007), respectively. Values are shown as average \pm SEM.

To do so, we analyzed a partial germ cell depletion model induced by a moderate dose of busulfan (10 mg/kg). In this condition, testis shows acute and massive germ cell death, causing the reduction of the number of GFR α 1+ units to a minimum of around one-third of the steady-state value by postinsult day 10. Then the number of GFR α 1+ spermatogonia gradually comes back to their preinsult level in about 2 months (Nakagawa et al., 2010).

Experimentally, we pulse labeled the GFR α 1+ cells on post-insult day 10 and analyzed their clonal fate in the following recovery phase (Figure 6A). Although the rate of Ngn3+ \rightarrow GFR α 1+ reversion increases substantially following insult (Nakagawa et al., 2010), the appearance of GFR α 1+ cells through this process was limited during the observed period (Figure S5A). As with steady state, in regeneration, the size of individual pulse-labeled clones diverged over time in the number of both constituent GFR α 1+ and GFR α 1– units (Figures 6B and 6C). Interestingly, a significant portion of clones lost all GFR α 1+ progeny even in regeneration. However, in contrast to steady state, the total number of GFR α 1+ units (viz. the average number of GFR α 1+ units per clone) increases because of a tilt in the overall balance of production of GFR α 1+ and GFR α 1– units toward the former (Figure 6D).

Then we questioned whether the same biophysical model could capture the clonal dynamics in regeneration, after seeding the lattice with GFR α 1+ units in proportion to their observed density and syncytial composition. As the simplest adaptation of the model, we allowed syncytial fragmentation to be uncompensated by loss of a neighboring GFR α 1+ unit, when the fragment migrates into a vacant domain. Under these conditions, we found that the predictions of the model showed a remarkable agreement with the measured clone dynamics, including the clone survival rate (Figure 6E), and the average and distribution of clone sizes (for both GFR α 1+ units[cells] and GFR α 1– units) (Figures 6D, 6F–6H, and S5B), over the wide range of time points for 18 days, if we made a minimal adjustment of the rate parameters. In particular, we found an optimal fit of the model to the data when the rates of cell division and fragmentation were both increased to around once per 8 days (from once per 10 days in steady state) and once per 10 days per bridge (from once per 20 days), respectively, while introducing a significant decrease of the death rate of GFR α 1– cells (once per 160 days, from once per 30 days). Using these parameters optimized from the short-term clonal behavior (up to 18 days), the model also accurately predicted the recovery in the tissue density of GFR α 1+ units and cells up to 2 months following insult, when the regeneration process largely completed (Figure S5C).

These results provide further support for the general validity and predictive power of the modeling scheme. Moreover, these results suggest that the dynamics of GFR α 1+ cells in regeneration following tissue injury is not based on a distinct program but follows the same pattern of stochastic rules as that seen in steady state.

DISCUSSION

Motivated by recent observations that question the validity of the prevailing “A_s model,” this study explored an alternative theory of the identity and behavior of mouse spermatogenic

stem cells and conducted single-cell-resolution analyses of the behavior of GFR α 1+ spermatogonia. The live-imaging study revealed that, in the GFR α 1+ compartment, practically all cell divisions are incomplete, whereas syncytial fragmentation occurs rather frequently, and that these processes follow constant rates that are independent of unit length. Based on these two measured rates, as well as cell density and localization in seminiferous tubules, we developed a minimal biophysical model to describe the dynamics of GFR α 1+ spermatogonia. This model could predict the range of complex data obtained from independent measurements, from the steady-state composition of GFR α 1+ units to the wide range of intricate clonal fate behaviors of pulse-labeled GFR α 1+ cells, both in steady state and in regeneration. Given the ability of such a highly simplified model to predict the complex in vivo behavior, we concluded that the principles that define the dynamics of GFR α 1+ compartment have been successfully resolved. On the other hand, the contribution of rare events, such as cell death or possible deviation from stochasticity, should be small enough to capture the overall dynamics of stem cells, although these factors should affect the detailed behavior of stem cells.

Figure 7A illustrates the stem cell dynamics proposed in this study, in which GFR α 1+ units continuously extend via incomplete division and fragment via intercellular bridge breakdown, while giving rise to Ngn3+ progeny. In this scheme, individual GFR α 1+ cells constantly change their state reversibly between single cells and variable lengths of syncytia; Figure 7B represents a typical example of such stochastic fate behavior predicted in silico. Yet, through this process at the population level, the number and composition of GFR α 1+ cells, as well as the production rate of Ngn3+ cells, were kept constant. Therefore, we propose that the entirety of GFR α 1+ spermatogonia comprises a single “stem cell pool.”

Onset of Ngn3 expression represents the exit from the stem cell compartment toward differentiation. Although this transition does not indicate the loss of reversibility, Ngn3+ cells show a pronounced differentiation bias. Indeed, in steady state, the vast majority of pulse-labeled Ngn3+ cells differentiate into spermatozoa and disappear from the tissue after a couple of months. When cells become Kit+, they appear to further decrease in potential to return into the GFR α 1+ compartment (Figure 7A, black broken arrows) (Barroca et al., 2009; Nakagawa et al., 2007, 2010). In parallel, the frequency of (incomplete) cell division increases, whereas syncytial fragmentation becomes more infrequent (Huckins and Oakberg, 1978; Nakagawa et al., 2010). As a result, the bulk of Ngn3+ and Kit+ spermatogonia extend their unit length unidirectionally as differentiation progresses (Figure 7A).

In mouse spermatogenesis, syncytial fragmentation was proposed based on the odd-numbered spermatogonial units observed after irradiation (Erickson, 1981) and directly filmed for the first time in Ngn3+ spermatogonia (Nakagawa et al., 2010). Given its low frequency in steady state, this process has been considered functionally significant in regeneration, where fragmentation appears to occur much more frequently (Nakagawa et al., 2010; Spradling and Fan, 2010). However, the current study revealed that, in the GFR α 1+ compartment, syncytial fragmentation regularly occurs in steady state at a frequency comparable with that of cell division and that this

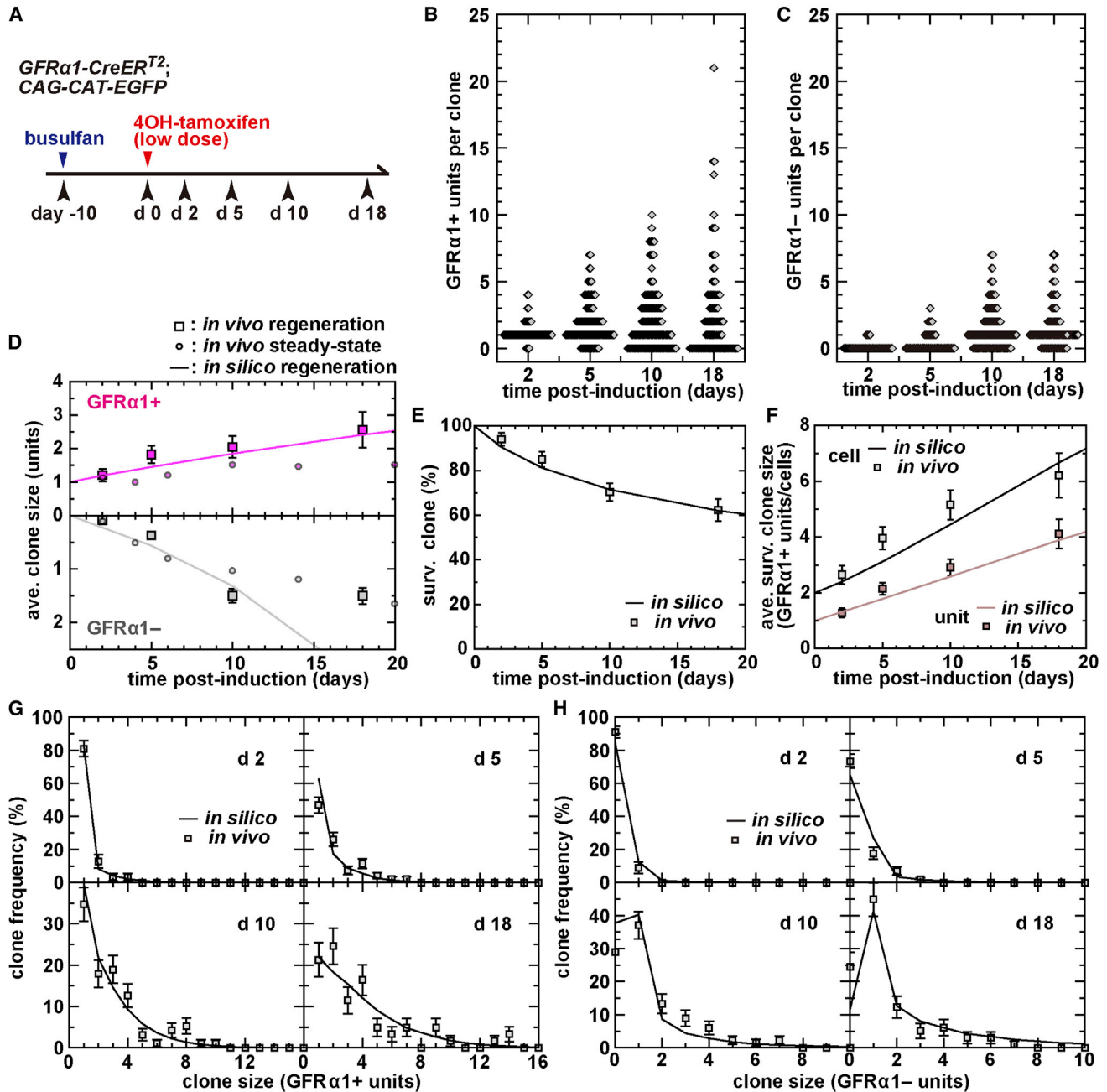


Figure 6. Dynamics of $GFR\alpha1+$ Spermatogonia during Regeneration

(A) Experimental schedule for clonal analysis of $GFR\alpha1+$ spermatogonia during regeneration.

(B and C) Distribution of clone size over 18 days after induction scored by the number of $GFR\alpha1+$ (B) and $GFR\alpha1-$ (C) units.

(D–H) The observed *in vivo* clonal fate behavior of the pulse-labeled $GFR\alpha1+$ units in regeneration (squares; shown as average \pm SEM), and their recapitulation by *in silico* model prediction after fitting for the rates of *D* and *F* and death of $GFR\alpha1-$ units (solid lines; see main text): average numbers of $GFR\alpha1+$ (upper) and $GFR\alpha1-$ (lower) units per clone compared with those in steady-state (small circles; reproduced from Figure 2L) (D), the percentage of surviving clones (E), the average number of $GFR\alpha1+$ unit(cell) per clone (F), and the clone size distribution scored by the number of $GFR\alpha1+$ (G) and $GFR\alpha1-$ units (H) (replotted from B and C, respectively).

process plays a fundamental role in the maintenance of stem cell pool and the continuity of spermatogenesis.

Within the modeling scheme established in this study, the maintenance of the $GFR\alpha1+$ population relies on the balance between their multiplication (syncytial fragmentation) and loss

(transition into $Ngn3+$), which are locally coordinated. This provides a cell-level explanation for the population asymmetry of mouse spermatogenic stem cells that was first discovered by large-scale (millimeters of patch length) and long-term (over months of chase period) clonal fate analysis of pulse-labeled

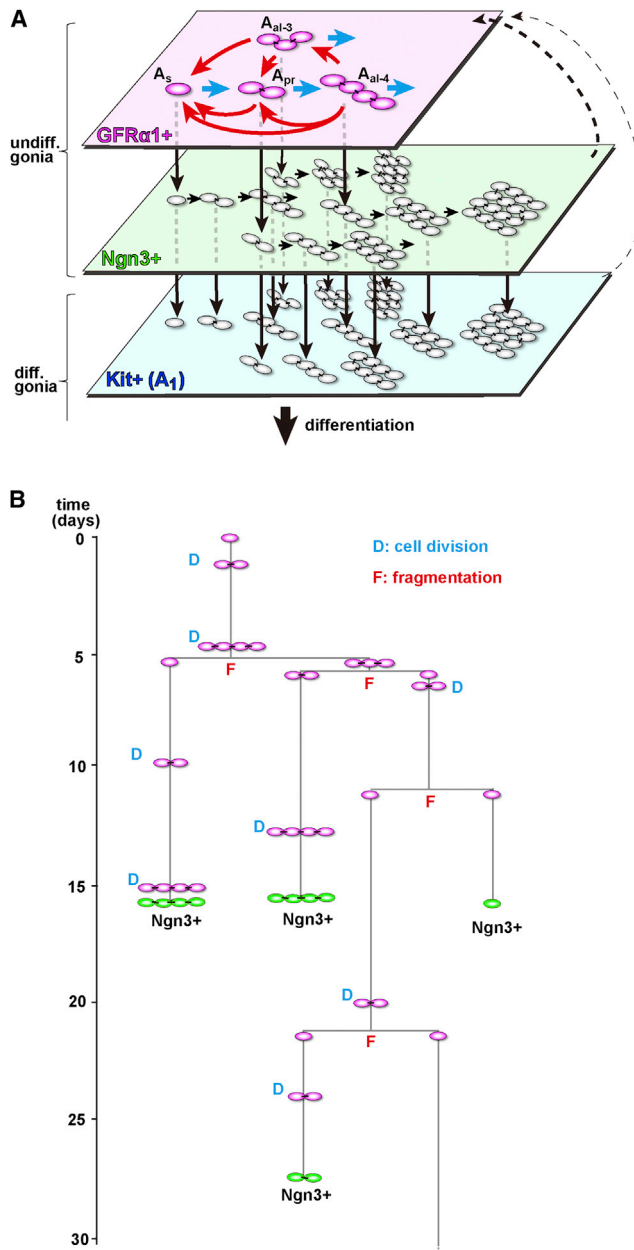


Figure 7. A Proposed Stem Cell Dynamics of Mouse Spermatogenesis

(A) A scheme of the proposed stem cell dynamics. On the top of the differentiation hierarchy, $GFR\alpha 1+$ spermatogonia comprise a single stem cell pool, in which cells continually and reversibly interconvert between states of A_s , A_{pr} , and A_{al} spermatogonia through incomplete cell division (blue arrows) and syncytial fragmentation (red arrows), while giving rise to $Ngn3+$ cells. After leaving the $GFR\alpha 1+$ compartment, differentiation-destined cells follow a series of transition ($GFR\alpha 1+ \rightarrow Ngn3+ \rightarrow Kit+$; downward black arrows) that accompanies the extension of syncytial length (rightward black arrows). $Ngn3+$ and, to a lesser extent, $Kit+$ cells retain the capacity to revert into the $GFR\alpha 1+$ compartment in a context-dependent fashion (broken arrows).

(B) Pedigree of a $GFR\alpha 1+$ unit-derived clone evolved in the *in silico* modeling scheme, representing a typical interconversion between A_s and syncytial states through incomplete cell division (D) and fragmentation (F), as well as generation of $Ngn3+$ spermatogonia.

$Ngn3+$ cells (Klein et al., 2010; Nakagawa et al., 2007). Supporting this idea, the rate of stem cell loss and replacement and the long-term scaling property of the clonal patch length distribution obtained by Klein et al. (2010) agree quantitatively with those predicted by the current biophysical model (Figure 5D; Supplemental Experimental Procedures).

In tissues without formation of syncytia, population asymmetry is typically achieved by local correlation of the division and loss (differentiation) of stem cells (Klein and Simons, 2011). In mouse spermatogenesis, the striking capacity of the model to capture the clonal fate behavior suggests that syncytial fragmentation may be linked with loss (transition to $Ngn3+$). However, the current study can address neither the causal relationship between fragmentation and loss nor the mechanism that coordinates these processes. To answer these questions, it is important to reveal the (yet unknown) mechanism that keeps the local density of $GFR\alpha 1+$ units constant in seminiferous tubules, a feature that was built into the construction of our current modeling scheme.

This study highlights a long-held question in germ cell research: what is the biological significance of intercellular connection? After meiosis, it is established that the connection ensures the formation of equivalent gametes from haploid spermatids, utilizing the shared cytoplasmic gene products, including those from X and Y chromosomes (Braun et al., 1989). However, the role of the connection remains an open question for diploid spermatogonia. Given the theory of equipotent stem cell pool composed of the entire $GFR\alpha 1+$ spermatogonia, the connection appears to be unrelated to their stem cell potential. However, $Ngn3+$ and $Kit+$ spermatogonia harbor more stable intercellular bridges, suggestive of some unknown role of intercellular connection in this differentiation-destined compartment. Further investigations are warranted to address this fundamental question.

Another important finding of this study is that the seemingly complex dynamics of the $GFR\alpha 1+$ compartment can be effectively described only by the rates of incomplete cell division (D) and syncytial fragmentation (F), the governing parameters of the modeling scheme. This notion is strengthened by the analysis of regeneration (Figure 6), which suggests that these rates are regulated in parallel throughout the $GFR\alpha 1+$ compartment, independent of their unit length, so that $GFR\alpha 1+$ spermatogonia can rapidly recover their pool size. Interestingly, the steady-state composition of $GFR\alpha 1+$ units with different lengths sensitively reflects the ratio of these rates, D/F (Figure 4D). In this scheme, the generation and frequency of spermatogonial units are simply a reflection of this ratio, including not only A_s , A_{pr} , A_{al-4} , A_{al-8} , etc., that are considered “regular” in the “ A_s model” but also those with 3, 5, 6, 7 cells, etc., that are often considered “irregular.” Together with the mutual interchange of these morphologically different states, we propose that all $GFR\alpha 1+$ units are equally regular and, in particular, that A_s is not a special entity. We also propose here the nomenclature of A_{al-3} , A_{al-5} , A_{al-6} , A_{al-7} , etc., without distinction from A_s , A_{pr} , A_{al-4} , A_{al-8} , etc.

Although the results of this study fully support the theory of a single stem cell pool comprised of functionally equivalent A_s and syncytial $GFR\alpha 1+$ spermatogonia (Figure 7A), one may also conjecture that a small population of slow-cycling $GFR\alpha 1+$ A_s cells that always undergo complete division act as

the “true” stem cells. If this were the case, then one would expect a transfer of the clonal dynamics from that represented by the behavior of short-lived cells (which interconvert between A_S and syncytia) to that of the slow-cycling A_S population over a long time course. However, such transfer was not observed in the fate behavior of $GFR\alpha 1+$ cells over extended timescales, from days to over a year. Therefore, although we do not rule out the presence of such a slow-cycling compartment, we can conclude that their contribution (would they exist) is not essential to maintain life-long spermatogenesis in mouse (for details, see the [Supplemental Experimental Procedures](#)). It remains open, however, in other animals with longer longevities, whether the same scheme of a single stem cell pool can be extrapolated to the years- or decades-long spermatogenesis or whether some slow-cycling population plays a significant role. Interestingly, primate testes host a large number of immature spermatogonia, A_{dark} , which appear not to have rodent counterparts ([Hermann et al., 2010](#)). Elucidating the roles of this population would be warranted to address this interesting question.

In this study, the live-imaging observation also revealed a unique property of $GFR\alpha 1+$ spermatogonia: the active movement over the seminiferous tubules. To orchestrate the local coupling of syncytial fragmentation and loss (transition into $Ngn3+$) of sparsely distributed $GFR\alpha 1+$ spermatogonia, it is vital that $GFR\alpha 1+$ cells are able to freely relocate. Failure to do so would elicit progressive unevenness in the local density of $GFR\alpha 1+$ cells, which would eventually compromise the integrity of the tissue. Indeed, the high motility of stem cells observed in mouse spermatogenesis may be paradigmatic of systems in which the stem cell niche is “facultative” or “open” ([Morrison and Spradling, 2008](#); [Stine and Matunis, 2013](#)). This shows a stark contrast with other systems supported by “definitive” or “closed” niche, such as *Drosophila* germline, mammalian intestinal crypt, or hair follicles, where stem cells lie in close association with each other and remain attached to a localized niche structure, and their movement must be limited within the niche region ([Fuller and Spradling, 2007](#); [Lin and Spradling, 1997](#); [Sheng and Matunis, 2011](#)). In future studies, it will be important to understand how the movement of stem cells is controlled and regulated by the interaction with such niche environments.

Finally, although incomplete division and syncytial fragmentation are germ cell specific, this study may provide important insights for other stem-cell-supported systems. In particular, we show that stem cells can be defined, not as a particular cell type, but as a heterogeneous population in which cells continually interconvert between different states. Indeed, such “dynamical heterogeneity” resonates with the recent live-imaging study of hair follicle stem cells, which show that self-renewal potential may be correlated with position within the stem cell niche ([Rompolas et al., 2013](#)).

EXPERIMENTAL PROCEDURES

Animals

$GFR\alpha 1^{CreERT2}$ knockin mice were generated as described in [Figure S1A](#). $GFR\alpha 1^{EGFP}$ ([Uesaka et al., 2007](#)), $CAG-CAT-EGFP$ ([Kawamoto et al., 2000](#)), and $GATA1-EGFP$ ([Suzuki et al., 2009b](#)) alleles were as previously described. All the mice used in this study were heterozygous for one or two of these alleles and simply indicated by their allelic name(s), with the background of C57BL/6 (from Japan CLEA and Japan SLC and used as wild-type animals).

Although mice carrying one nonfunctional knockin allele of the $GFR\alpha 1$ gene, $GFR\alpha 1^{CreERT2}$, and $GFR\alpha 1^{EGFP}$ were used for pulse-labeling and live-imaging experiments, respectively, such heterozygosity neither affected the total density and composition of $GFR\alpha 1+$ units nor the overall integrity of spermatogenesis, over 1 year postlabeling ([Figures S1D–S1I](#)). All animal experiments were conducted with approval of The Institutional Animal Care and Use Committee of National Institutes of Natural Sciences, unless specifically mentioned.

Whole-Mount Immunofluorescence of Seminiferous Tubules

Immunostaining of whole-mount seminiferous tubules was performed as previously described ([Nakagawa et al., 2010](#)) using anti- $GFR\alpha 1$ Ab (1:1,000 dilution; R&D Systems), anti-GFP Ab (1:300 dilution; Invitrogen), and anti-Kit Ab (1:200 dilution; BD Biosciences). Observation and photography were performed with a BX51 upright fluorescence microscope equipped with a DP72 CCD camera (Olympus). Spermatogonia were judged as belonging to a syncytium when, based on a continuous $GFR\alpha 1$ or GFP staining using a 60× water immersion objective lens, the cell-cell connection was visually detected. To measure the lengths of the patches of GFP+ cells, M205C stereomicroscope with a DFC490 CCD camera (Leica) was used.

Pulse Labeling of $GFR\alpha 1+$ Spermatogonia

Three-month-old $GFR\alpha 1-CreER^{T2}$; $CAG-CAT-EGFP$ mice were injected intraperitoneally with 2.0 mg (for a higher labeling efficiency) or 0.25–0.35 mg (for clonal labeling) per individual of 4OH-tamoxifen (Sigma) dissolved in ethanol, in dimethyl sulfoxide, and then in sesame oil (Nakalai Tesque). For clonal lineage tracing, their testes were processed for whole-mount immunofluorescence. To induce regeneration, mice were intraperitoneally injected with busulfan (10 mg/kg) as described previously ([Nakagawa et al., 2010](#)) prior to 4OH-tamoxifen administration.

Intravital Live Imaging

Live-imaging of the testes of 4- to 5-month-old $GFR\alpha 1^{EGFP}$ or $GFR\alpha 1^{EGFP}$; $GATA1-EGFP$ mice under anesthesia was performed as described before, using epifluorescence IX61WI microscope (Olympus) ([Yoshida et al., 2007](#)). Time-lapse images were captured at the rate of one frame per 30 min using the Andor iXon EM-CCD camera controlled by Metamorph software (Molecular Devices). Movies were constructed by Metamorph software, and the trajectories of spermatogonia and Sertoli cells were manually extracted from movies using Metamorph and ImageJ software. An intercellular bridge was deemed to be intact if the cells remained within 30 μ m for more than 12 hr ([Supplemental Experimental Procedures](#)). Although only $GFR\alpha 1+$ cells located in the superficial region of the testis were filmed, their representativeness is supported by the agreement of the densities and compositions of $GFR\alpha 1+$ units measured by live-imaging and whole-mount immunostaining studies (the latter represents the average values of the entire tubules) ([Figures S3C–S3E](#)). It is further consolidated by the correspondence between rates of cell division and syncytial fragmentation measured from live-imaging data and the range of fate behavior of pulse-labeled $GFR\alpha 1+$ cells found evenly throughout the testis (see text).

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes Supplemental Experimental Procedures, five figures, one table, and five movies and can be found with this article online at <http://dx.doi.org/10.1016/j.stem.2014.01.019>.

AUTHOR CONTRIBUTIONS

K.H., B.D.S., and S.Y. designed the experiments, performed data analysis, and wrote the manuscript. K.H. and T.N. performed *in vivo* experiments. B.D.S. performed *in silico* analyses. H.E., M.S., and M.Y. generated genetically modified animals.

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