

Capacity for stochastic self-renewal and differentiation in mammalian spermatogonial stem cells

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Mammalian spermatogenesis is initiated and sustained by spermatogonial stem cells (SSCs) through self-renewal and differentiation. The basic question of whether SSCs have the potential to specify self-renewal and differentiation in a cell-autonomous manner has yet to be addressed. Here, we show that rat SSCs in ex vivo culture conditions consistently give rise to two distinct types of progeny: new SSCs and differentiating germ cells, even when they have been exposed to virtually identical microenvironments. Quantitative experimental

measurements and mathematical modeling indicates that fate decision is stochastic, with constant probability. These results reveal an unexpected ability in a mammalian SSC to specify both self-renewal and differentiation through a self-directed mechanism, and further suggest that this mechanism operates according to stochastic principles. These findings provide an experimental basis for autonomous and stochastic fate choice as an alternative strategy for SSC fate bifurcation, which may also be relevant to other stem cell types.

Introduction

In mammalian testes, spermatogonial stem cells (SSCs) sustain continuous spermatogenesis throughout a mammal's reproductive years. The power of SSCs depends on their ability to produce two types of progeny: one type replicates the mother stem cell (self-renewal); the other acquires specialized function and morphology to become sperm (differentiation). Although no known molecular markers can unambiguously identify SSCs, it is generally accepted that SSCs abut the basement membrane of the seminiferous tubules as single cells (A_{single}). The earliest identifiable differentiating progeny are sibling spermatogonia (A_{paired}) that do not complete cytokinesis but form intercellular bridges, which connect their cytoplasm into a syncytium (Huckins, 1971; Oakberg, 1971; de Rooij and Grootegoed, 1998; Oatley and Brinster, 2006, 2008). A_{paired} then develop into A_{aligned} (chains of 4, 8, 16, and 32 cells) through a series of synchronous mitotic divisions with incomplete cytokinesis. Subsequently, A_{aligned} undergo a lengthy differentiation process

that eventually leads to meiosis and formation of haploid spermatids (Russell et al., 1990).

How SSC progeny adopt different cell fates has not been elucidated. Most current models presume a causal correlation between particular environmental cues and a specific cell fate outcome. For instance, in the niche model, it is proposed that a stem cell can only self-renew within a specialized microenvironment (niche) that promotes "stemness" and prevents differentiation, whereas differentiation only occurs outside the niche environment (Schofield, 1978; Spradling et al., 2001). Support for the niche model comes from many studies of diverse tissue stem cells in various organisms (Spradling et al., 2001; Fuchs et al., 2004; Li and Xie, 2005), especially in *Drosophila* testis, where the niche constituents as well as their detailed role in SSC fate determination have been characterized (Gilboa and Lehmann, 2004; Yamashita and Fuller, 2005; Fuller and Spradling, 2007). However, within mammalian seminiferous tubules, SSCs are intermingled with differentiating germ cells as a monolayer on the

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Dr. Garbers died on 5 September 2006.

Abbreviations used in this paper: GCS, germ cell-specific; GDNF, glial cell line-derived neurotrophic factor; MEF, mouse embryonic fibroblast; SSC, spermatogonial stem cell.

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basement membrane, where no specialized niche has been found (Ogawa et al., 2005). Recently, a few studies suggest that peritubular blood vessels or interstitial cells might serve as niches (Chiarini-Garcia et al., 2001; Yoshida et al., 2007). However, due to their much larger scale relative to individual germ cells and the lack of direct contact with them, the peritubular structures are unlikely to differentially influence the two intermingled cell types to account for alternative fate specification, and their role in SSC self-renewal and differentiation remains to be examined.

An outstanding question is whether mammalian SSCs are able to specify self-renewal and differentiation cell autonomously and independently of differential extrinsic stimuli. Evaluating this possibility is essential for determining the causes and mechanisms governing SSC alternative fate decisions. Autonomous fate choice can be tested through determining if mammalian SSCs can give rise to both SSCs and differentiating germ cells under identical environmental conditions. We therefore characterized the fate outcomes of rat SSC daughter cells in a well-characterized homogeneous *ex vivo* culture setting that supports expansion of rodent SSCs and preserves their ability to generate offspring upon transplantation back into testes (Hamra et al., 2005; Kanatsu-Shinohara et al., 2003, 2005a; Kubota et al., 2004b; Ryu et al., 2005; Wu et al., 2009).

The culture method employs fibroblast feeder cells and defined serum-free media supplemented with glial cell line–derived neurotrophic factor (GDNF), a growth factor essential for SSC survival/proliferation *in vivo* (Meng et al., 2000). The presence, quantity, and physiological properties of SSCs in these cultures have been assessed by the testis cell transplantation assay, a functional and quantitative SSC assay previously developed by Brinster and co-workers (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994; Brinster, 2002). In this assay, germ cells are transplanted into seminiferous tubules that are depleted of endogenous germ cells, and only donor SSC-derived progeny develop as discrete colonies of spermatogenesis over time. Because each colony typically arises through the self-renewal and differentiation of a single stem cell, the resulting colony number offers a quantitative measure of SSC content (Dobrinski et al., 1999; Nagano et al., 1999; Brinster, 2002; Zhang et al., 2003; Kanatsu-Shinohara et al., 2006; Oatley and Brinster, 2006). Using this assay, the cultured SSCs have been shown to repopulate recipient testes and produce functional sperm, indicating that their potential for both self-renewal and differentiation are fully preserved. Furthermore, SSC number continues to increase as the total germ cell population expands over time; hence, SSC self-renewal is taking place in culture (Kanatsu-Shinohara et al., 2003, 2005a; Kubota et al., 2004b; Hamra et al., 2005; Ryu et al., 2005; Wu et al., 2009). However, it is not clear if self-renewal is the only outcome of SSC divisions or whether differentiation is also taking place in the cultures.

Here, we find that under the established uniform culture conditions, SSCs consistently give rise to both new SSCs and differentiating progeny. The differentiating progeny have lost stemness and unlimited self-renewal capacity but have developed functional intercellular bridges, forming syncytia among sibling cells similar to A_{pair} and A_{aligned} in testis. Twin daughter cells of single SSCs often undergo self-renewal and differentiation side by side even though they have been exposed to virtually

identical microenvironments. Moreover, quantitative experimental measurements and mathematical modeling indicates that fate decision is stochastic, with constant probability. These results demonstrate the capacity of a mammalian SSC to specify both self-renewal and differentiation autonomously, and this capacity operates according to stochastic principles.

Results

Germ cells in culture remain heterogeneous in terms of stemness and proliferation potential

If self-renewal and differentiation are ordained by environmental cues, a uniform environment should result in uniform cell fate specification (Fig. 1 A, left). In contrast, if fate decision is intrinsic to the cell, different fates could arise in a homogeneous culture (Fig. 1 A, right). To distinguish between these two scenarios, we first determined whether all the rat germ cells in culture are SSCs. Previous studies have shown that cultured rat germ cells remain heterogeneous during the first few months outside the testes (Hamra et al., 2005; Ryu et al., 2005). To examine whether this was due to transient proliferation of differentiating germ cells from testes, we derived germ cell lines from rat testes that express EGFP specifically in all germ cells (Cronkhite et al., 2005), and cultured them continuously for much more extended periods. Even after 20 mo of propagation, SSC content in the cultures remained $\sim 17\%$ of the total germ cells, as measured by the transplantation assay (Fig. 1 B), which is similar to the initial reports (Hamra et al., 2005; Ryu et al., 2005). Thus, besides SSCs, a non-stem germ cell population persists and constitutes a significant fraction ($>80\%$) of the culture, which suggests that they either have unlimited proliferation capacity or are continuously replenished by an immortal cell type, presumably the SSCs.

We next examined whether the proliferation potential of the germ cells in culture is also heterogeneous. Because the cultures have been shown to expand indefinitely (Kanatsu-Shinohara et al., 2003; Kubota et al., 2004a; Hamra et al., 2005; Kanatsu-Shinohara et al., 2005a, 2008; Ryu et al., 2005), and offspring can be produced from single SSC-derived long-lasting clones (Kanatsu-Shinohara et al., 2005c), it is evident that at least some of the germ cells, especially some SSCs, must be immortal in culture. Nevertheless, we observed widespread and continued cell death in culture through long-term time lapse imaging (Fig. S1); only $\sim 18\%$ ($n = 221$) of identified single germ cells gave rise to clones that continued to proliferate beyond 2 wk. To confirm this and to measure the longevity of the mortal cells, single EGFP-positive germ cells were sorted into 96-well plates and cultured. We found that $21.0 \pm 4.1\%$ (mean \pm SEM) of these single cells continue to proliferate for at least 6 mo and can be considered as immortal, whereas the other 79% proliferate only transiently and form small clones (Fig. 1 B). Of the transient clones, 91.8% perish within the first two weeks. TUNEL staining indicates that the dying cells contain double-strand DNA breaks (Fig. 1 C), which suggests that their death is by apoptosis. Because unlimited proliferation potential is a defining attribute of stem cells, the similar percentages of immortal cells and SSCs implies that all the immortal cells are SSCs and the non-stem cells are mortal.

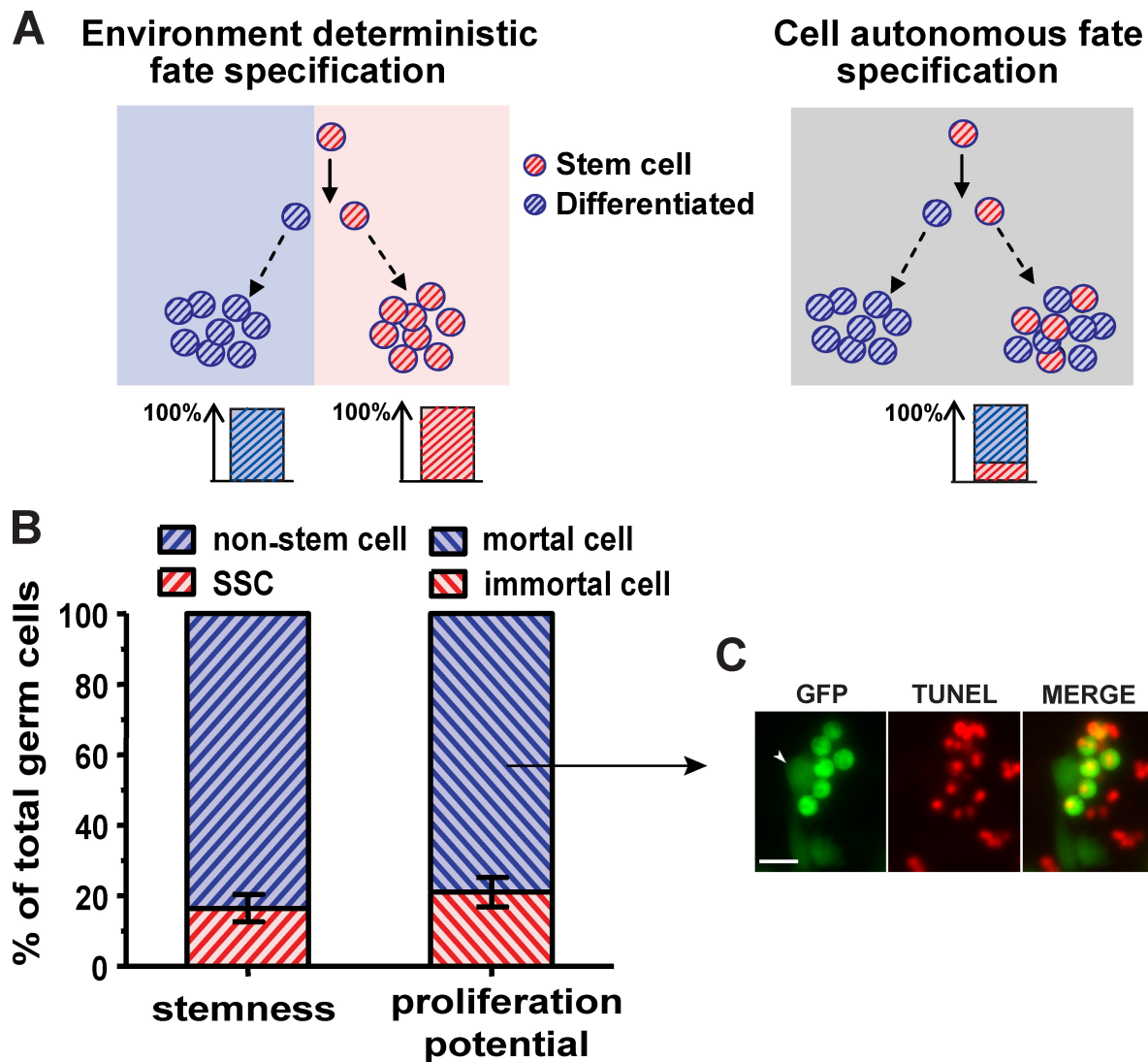


Figure 1. **Persistent heterogeneity of germ cells in stemness and proliferation potential under uniform culture conditions is compatible with a cell-autonomous fate specification model.** (A) Environment deterministic and cell-autonomous fate specification models predict homogeneous and heterogeneous composition of SSC progeny, respectively, in a uniform environment. (A, left) In the environment deterministic model, self-renewal and differentiation are controlled by distinct environmental cues (red and blue), which result in homogeneous yet distinct fate outcomes in the red and the blue area. (A, right) In the cell-autonomous fate specification model, occurrence of self-renewal and differentiation does not depend on differential environmental cues, and both SSCs and differentiating progeny can be generated in a uniform environment (gray). (B) Transplantation of germ cells that have been cultured for 20 mo into sterile testes shows that only $16.5 \pm 3.9\%$ (mean \pm SEM, $n = 6$) of the population are SSCs, and that 83.5% do not possess stemness. Tracing of sorted single germ cells in culture for 6 mo shows that $79.0 \pm 4.1\%$ (mean \pm SEM, $n = 84$ in three independent experiments) of the cells perish within a month after transient proliferation, and only 21.0% of the single cells can continue proliferate for at least 6 mo. (C) Dying germ cells show condensed cell bodies and are positive for TUNEL staining. The arrowhead points to a healthy TUNEL-negative germ cell that remains flattened on the culture substrate. Bar, 20 μ m.

Immortal germ cells are SSCs, nonstem germ cells are mortal

To investigate whether all the immortal cells are SSCs, we randomly selected 26 of the single cell-derived long-lasting clones and used the transplantation assay to determine whether they contain SSCs (outlined in Fig. 2A). Given that only a stem cell can give rise to stem cells, a positive result would indicate retrospectively that the founding cell of the clone was an SSC. From each clone, $\sim 5\text{--}25 \times 10^3$ cells were transplanted into individual rat recipient testes. 14 testes were analyzed 2 mo later, and the other 12 testes were examined 5 mo after transplantation. We found that 100% of the 26 transplanted clones had colonized the recipient seminiferous tubules, forming between 2 and 50 EGFP-positive colonies per

testis. A representative recipient testis is shown in Fig. 2B. This result suggests that all immortal cells are SSCs. Conversely, the non-stem cells are mortal, for they never form long lasting clones.

The mortal nonstem germ cells develop functional intercellular bridges, a conserved marker for germ cell differentiation

We next investigated whether the mortal non-stem cells have differentiated. Time-lapse imaging revealed that essentially all the mortal non-stem cells (>99%; Table S1) exhibited highly synchronized cell divisions and deaths as sibling groups (Fig. 3, A and B; and Video 1). Coordinated cell cycle and apoptosis have been extensively documented for differentiating germ cells

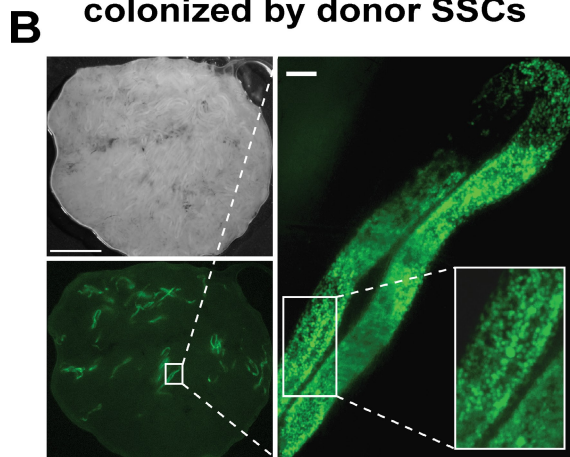
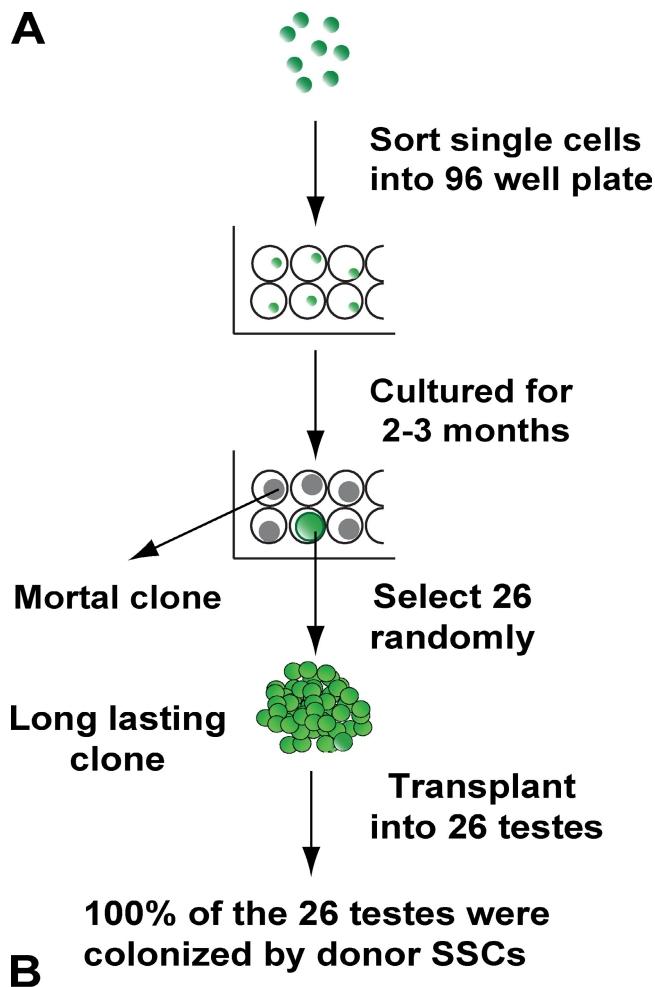


Figure 2. Immortal germ cells are SSCs. (A) Outline of the experimental protocol to yield single cell-derived long-lasting clones and subsequent transplantation of cells from these clones into presterilized recipient rat testes. (B) A wild-type recipient testis transplanted with a long-lasting clone derived from single EGFP-positive germ cell. The donor SSC-derived EGFP-positive spermatogenic colonies (left) indicate the founding cell of the long-lasting clone was an SSC. An enlarged view of individual donor SSC derived colonies (right) showed multiple layers of differentiating germ cells. This testis is representative of the 26 recipient testes, all of which were colonized similarly by donor SSCs from long-lasting clones. White boxes indicate sections that have been enlarged. Bars: (left) 500 μ m; (right) 100 μ m.

in rodent testes and have been shown to be mediated by intercellular bridges that connect the cytoplasm of sibling cells into a syncytium (Fawcett et al., 1959; Fawcett, 1961; Huckins, 1978; Hamer et al., 2003). Therefore, the synchrony observed in culture suggests that the mortal non-stem cells have formed intercellular bridges, a marker for differentiating germ cells that is conserved from invertebrates to human.

Consistent with this notion, germ cells in culture often appear to be interconnected by thin strands, like beads on a string (Fig. 3 D, arrows). To test for cytoplasmic continuity among these cells, we developed a novel assay specific for intercellular bridges using FRAP. In this assay, as outlined in Fig. 3 C, EGFP is photo-bleached specifically in one cell of a group, and the fluorescence intensities of all cells in the group are monitored by time-lapse imaging. If the cells are interconnected, diffusion should eventually result in equilibration of EGFP fluorescence intensity throughout the group. Because EGFP is too large to pass through gap junctions, the only other known junction that allows molecule exchange between mammalian cells, this assay is specific for intercellular bridges, which are usually 1–3 μ m in diameter (Weber and Russell, 1987; Greenbaum et al., 2007).

When this method was applied in culture, in most cases, we observed EGFP fluorescence recovery in the bleached germ cell accompanied by a corresponding fluorescence decrease in its contiguous neighbors, eventually reaching equilibrium (Fig. 4 D and Video 2). As a control, when EGFP in isolated cells that have no neighbors was photobleached, the fluorescence intensity in these cells never recovered significantly within a similar time frame (unpublished data). Hence, the majority of the germ cells in culture are coupled by intercellular bridges, which suggests that they are differentiating. This is consistent with the results from the transplantation assay showing that most germ cells in culture are not SSCs (Fig. 1 B). We also found a small fraction of the germ cells that do not exchange EGFP with any contiguous cells (Fig. 3 E), which indicates that differentiation has not been initiated or has not progressed as far in these cells. Therefore, some of these uncoupled cells are probably SSCs, in agreement with morphological observations *in vivo* indicating that SSCs are not interconnected with other cells (Huckins, 1971; Oakberg, 1971; de Rooij and Grootegoed, 1998).

To compare the intercellular bridges formed in culture with those formed *in vivo*, seminiferous tubules containing EGFP-positive spermatogonia were freshly dissected, and germ cell chains ($A_{aligned}$) on the basement membrane were subjected to the FRAP assay (Fig. 3 F). The kinetics of EGFP exchange in testis were indistinguishable from those observed in culture (Fig. 3, compare D and F), which suggests that the intercellular bridges formed in culture and in testes are physiologically comparable. Therefore, the mortal non-stem cells in culture appear to have initiated the same differentiation process as differentiating germ cells *in vivo*.

SSCs both self-renew and differentiate in culture

Given that the lifespan of the differentiating germ cells does not exceed 1 mo, their persistence in culture over many months implies that they must be continually replenished by the immortal SSCs. To verify this, we examined whether long-lasting clones

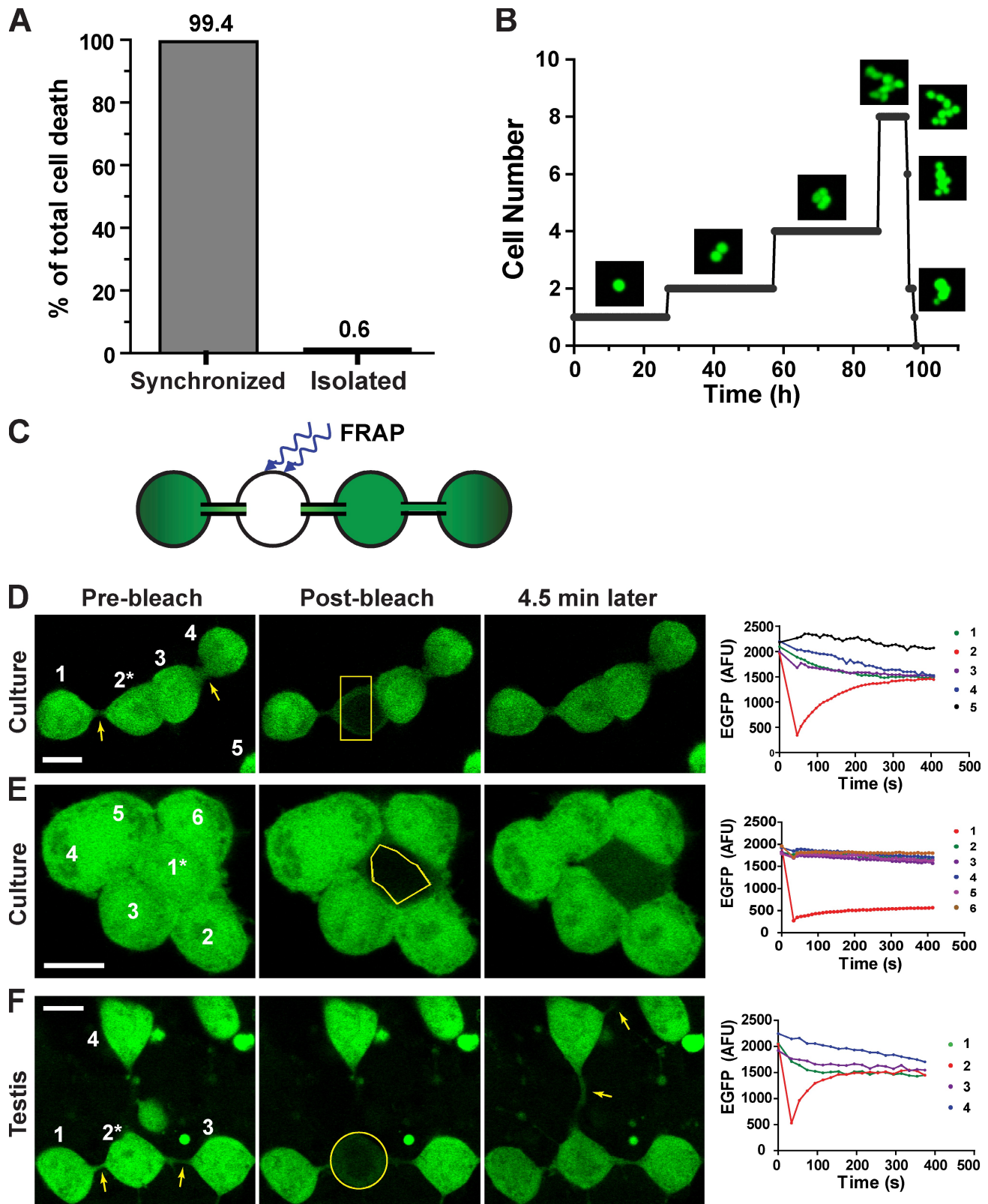


Figure 3. **Mortal nonstem germ cells in culture are differentiating.** (A) Analysis of time-lapse image sequences showed that among germ cells that have divided at least once in culture, 99.4% of the cell deaths ($n = 314$) are synchronized among sibling groups; only 0.6% died as isolated cells. (B) Images from a time-lapse sequence of a typical mortal germ cell-derived clone (Video 1) and a plot of the cell number within the clone over time show the synchrony of cell divisions and cell death of sibling germ cells in the clone. (C) The experimental scheme for a functional assay specific for intercellular bridges. It involves photobleaching of EGFP within one cell of a group and monitoring of EGFP fluorescence intensity of each cell by time-lapse imaging. (D–F) Time-lapse image sequences before and after photobleaching of the cell indicated by an asterisk, and a time trace of EGFP fluorescence intensity in each cell of a germ cell group in culture (D and E, also see Video 2) or in testis (F). Bleached regions are delineated by yellow lines. Arrows point to visible intercellular bridges. The numbers in D–F indicate which cells correspond to the fluorescence intensity traces shown on the right. Bars, 10 μm .

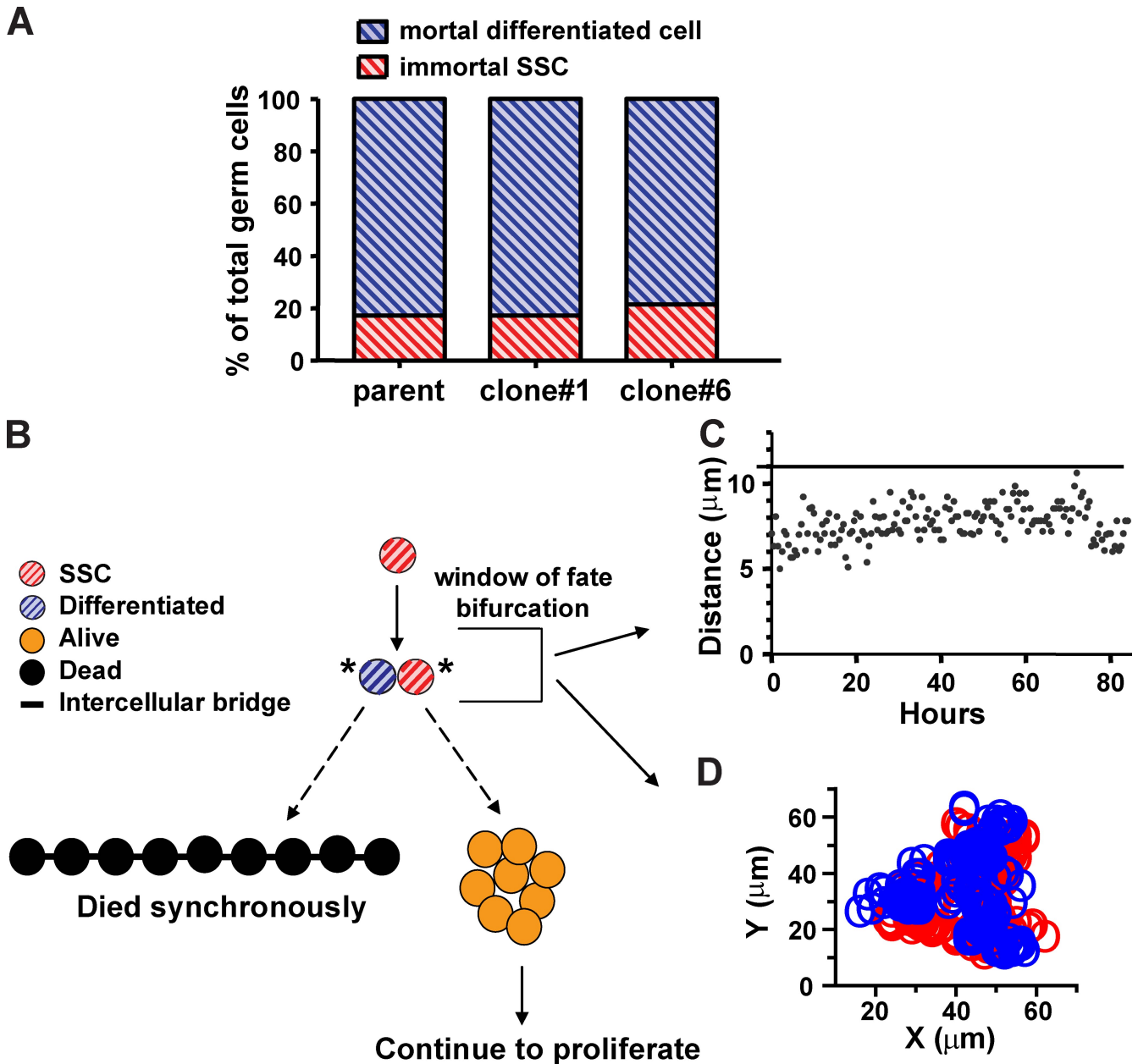


Figure 4. **Individual SSCs consistently give rise to SSCs and differentiating germ cells independently of environmental cues.** (A) Cultures of single germ cells from two randomly selected long-lasting clones (Nos. 1 and 6) derived from single SSCs showed that they contained 82.7% and 78.4% mortal differentiating cells and 17.3% and 21.6% immortal SSCs, respectively ($n = 84$). This was similar to their parent cell line (82.7% differentiating and 17.3% SSC, $n = 84$), which indicates that individual SSCs consistently give rise to both SSCs and differentiating germ cells. (B) Schematic diagram based on the long-term time-lapse imaging of twin daughter cells of a single SSC that gave rise to two clones with different fates. In one clone, all cells died synchronously by day 10, whereas the other continued to live by day 18, which indicates that one of the twin daughter cells differentiated, whereas the other became an SSC. (C and D) Relative positions of the two daughter cells during the time between their birth and next division (Video 3). (C) The solid line indicates the mean diameter ($\sim 13 \mu\text{m}$) of the two cells, and black dots mark the distance between the geometric centers of the two cells at the indicated time points. (D) Centroid positions of each daughter cell during the time window are represented by the centers of the red and blue circles, respectively. The diameter of the circles is equal to the mean diameter of the cells to show the area transiently visited by the two cells.

derived from single SSCs contain a mortal population. When single germ cells from these clones and from their parent lines were sorted into 96-well plates and cultured in parallel, a similar proportion of mortal cells were observed in each of them (Fig. 4 A).

In addition, by long-term time-lapse imaging, we also directly observed fate bifurcation in clones derived from single SSCs. In these experiments, SSCs were identified retrospectively

by their long-lasting progeny (>17 d), and differentiating germ cells were identified by progeny that formed intercellular bridges and died synchronously. We observed examples in which twin daughter cells of single SSCs acquired different cell fates, and others in which they both became SSCs. However, the method cannot unambiguously distinguish between an SSC that gives rise to two differentiating daughter cells and a mother germ cell that is already differentiating.

Within the SSC lineages observed, no obvious pattern of cell fate outcome was identified. However, divergence of SSC daughter cell fates, similar to the example diagrammed in Fig. 4 B, was always observed (100%, $n > 50$). Based on these observations, we concluded that individual SSCs not only undergo self-renewal, but also give rise to differentiating germ cells in culture.

Because under certain conditions differentiating germ cells can de-differentiate and become SSCs in *Drosophila* (Brawley and Matunis, 2004; Cheng et al., 2008) and mouse testes (Nakagawa et al., 2007; Barroca et al., 2009; Yoshida, 2009), we therefore examined if such fate conversion occurs in rat germ cell cultures. Even though the majority of rat germ cells in culture are c-kit negative (>98%; Fig. S2), which indicates an early state of differentiation, none of the cells identified as differentiating germ cells by our criteria gave rise to long-lasting clones ($n > 200$ cells). Consistently, in the SSCs examined ($n > 50$), none of them were derived from a cell that seemed to be connected to other cells through intercellular bridges. These observations indicate that de-differentiation, if it occurs at all in the culture, is rare.

SSC self-renewal and differentiation are independent of extrinsic heterogeneity

The fact that self-renewal and differentiation occur concomitantly in a uniform culture setting suggests an independence of cell fate choice with respect to the environment. However, it is formally possible that fate bifurcation is dependent on microscale heterogeneity associated with the feeder cells or extracellular matrix. Although direct and thorough evaluation of microscale heterogeneity is impossible, a detailed analysis of germ cell behaviors in culture suggests it is unlikely to be the reason for fate bifurcation. We found that SSC daughter cell pairs, including those that have adopted different fates, typically remained closely associated the whole time from their birth until they divided again to give rise to clones of either identical or distinct fates. This observation was quantified by measuring the distance between the geometric centers of twin daughter cells from time-lapse recordings. In most cases, as for the cell pair indicated by an asterisk in Fig. 4 B, the center-to-center distance was always less than their mean diameter, which indicated that the two cells were never separated into discrete microenvironments (Fig. 4 C and Video 3). Furthermore, twin daughter cells in all cases, instead of statically occupying one spot, constantly move around together, dynamically probing the same microenvironment. Typically, the areas transiently visited by each daughter cell during the fate bifurcation time window are nearly identical, as shown in Fig. 4 D. The intimate association and the dynamic movement of the two cells minimize the chances for their exposure to different extrinsic cues, including microheterogeneity. Nevertheless, as in this case, twin daughter cells derived from a single SSC often adopted different fates. Therefore, fate bifurcation does not seem to depend on extrinsic heterogeneity. Instead, cell-autonomous fate specification is most consistent with our data.

SSC self-renewal and differentiation are stochastic

Because SSC self-renewal results in continued proliferation, whereas differentiation leads to cell death in culture, fate specification will directly influence germ cell population growth,

death, and composition. We noted that the proportions of SSC and differentiating cells in the parent cultures and in long-lasting clones derived from single SSCs are essentially invariant (Fig. 4 A), which suggests a constant ratio of SSC self-renewal and differentiation. In addition, we did not observe any consistent pattern of fate outcome along the lineage trees of long-lasting clones. These observations suggest that self-renewal and differentiation might be stochastic events occurring with a consistent probability. Based on this premise, we derived the following mathematical models for the kinetics of total germ cell growth (Eq. 1) and for the decay of single cell-derived clones over time (Eq. 2, detailed in Materials and methods):

$$\ln G_t = [\ln(2s)/c]t + \ln G_0. \quad (1)$$

And when $t < (q + 1)c_{max}$,

$$P_{alive} = -c S_0 (1-s) s^{-(q+1)} / [G_0 \ln(s)] s^{t/c} + S_0 / G_0, \quad (2)$$

where G_t denotes the number of total live germ cells at time t , S_0 denotes the number of SSCs of the given germ cell population at arbitrary time zero during continuous culture, s denotes the chance per cell division that an individual SSC daughter cell will become an SSC. The probability that it will become differentiated is $1 - s$, and q denotes the number of cell divisions needed for newly differentiated germ cells to produce the mean number of progeny before their death. c denotes mean cell cycle length (40.0 ± 8.2 h), which is not different for SSC and differentiating germ cells as measured by long-term time-lapse imaging (Fig. 5 A). c_{max} denotes the observed maximum cell cycle length, which is 86 h. P_{alive} denotes percentage of live clones derived from single germ cells of a given population at time t .

These equations assume that SSCs are immortal and that differentiating germ cells die after transient propagation, as we have shown experimentally.

Eq. 1 fits the measured total germ cell growth well when $s = 0.706$ ($r^2 = 0.9987$; Fig. 5 B), and Eq. 2 fits the measured survival curve of single cell-derived clones when $s = 0.618$ and $q = 3.3$ ($r^2 = 0.9720$; Fig. 5 C). These analyses thus showed that the probabilistic model can coherently describe various uncorrelated aspects of population dynamics in culture. The analyses also revealed that the probability for SSC self-renewal under the particular culture conditions is $\sim 67\%$ (mean of 0.706 and 0.618); for differentiation, the probability is 33%. Even though fate decisions are biased toward self-renewal, differentiating germ cells actually outnumber the SSCs in culture because 100% of their progeny will be differentiated, whereas the SSC progeny will be a mixture of SSCs and differentiating progeny.

In addition, according to the model, the stem cell fraction in culture is:

$$R_s = s^{(q+1)}, \quad (3)$$

which is not a function of time (see Materials and methods). Therefore, the model predicts that the stem cell ratio in culture should remain constant, with a value of 17.9% (assuming $s = 0.67$ and $q = 3.3$). To verify this prediction, we used the

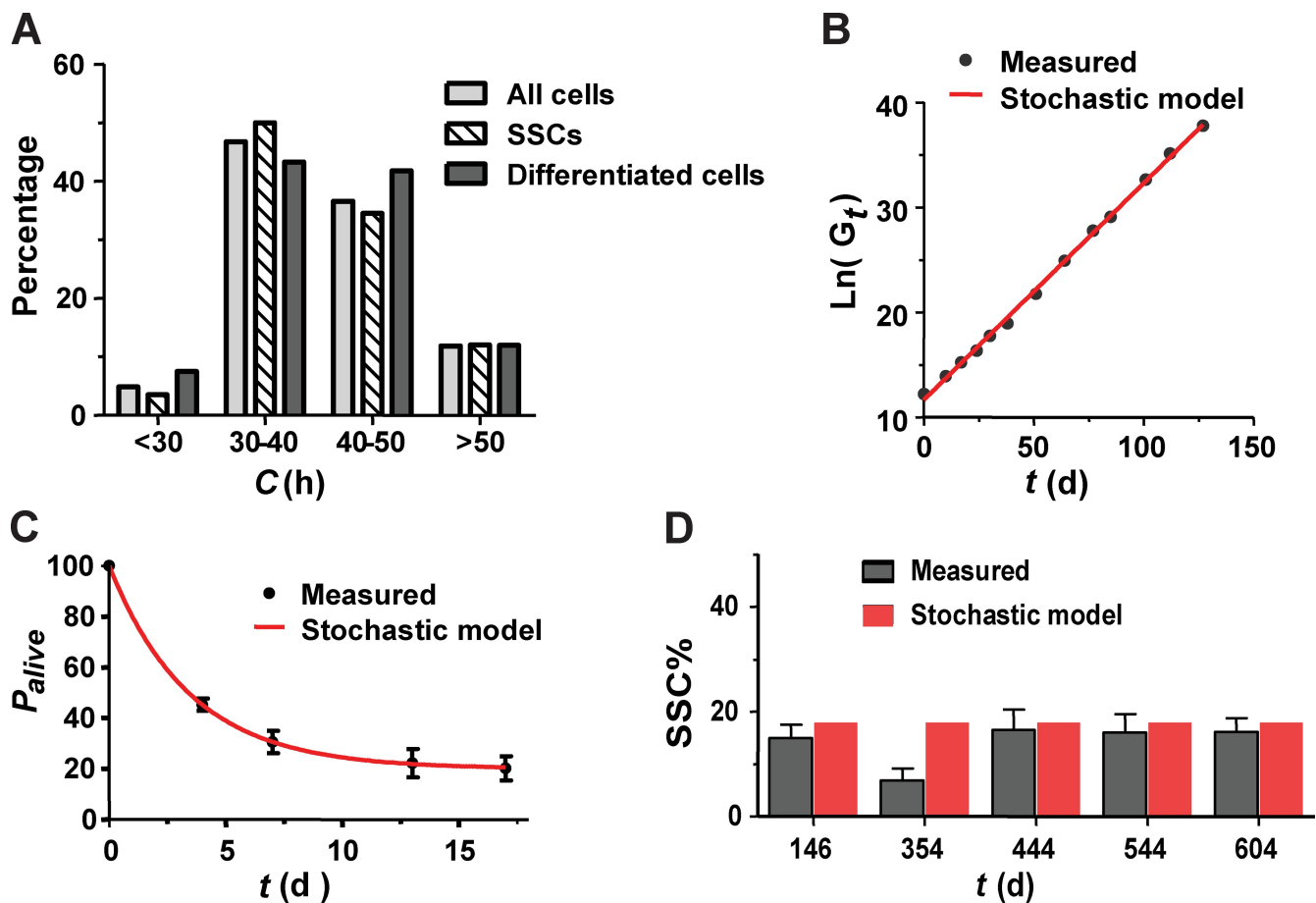


Figure 5. **A mathematical model for stochastic fate specification describes and predicts germ cell population dynamics in culture.** (A) Histogram of cell cycle length in groups of retrospectively identified SSCs ($n = 110$), differentiating mortal cells ($n = 76$, synchronized sibling divisions are counted as one), and all cells ($n = 186$). No significant difference was observed between the SSCs and the differentiating cells. Mean cell cycle length for all cells was 40.0 ± 8.2 h. (B and C) The mathematical model (red line) fits the measured germ cell growth (B, black dots), and the measured germ cell survival curve (C, black dots; mean \pm SEM, $n = 84$ in three independent experiments). (D) The mathematical model predicts a constant SSC content of 17.9% over time (red bars). Dark gray bars indicate the measured SSC content at the indicated time points (mean \pm SEM, $n > 4$).

transplantation assay to measure the number of SSCs in continuous cultures of several independently derived germ cell lines over extended periods of time. One set of such experiments is shown in Fig. 5 D. These measurements confirmed that the SSC fraction remains essentially constant over time. The measured SSC percentages are also comparable to the predicted 17.9%. Hence, our data fully support the model of stochastic fate determination with constant probability.

Discussion

Further discussion of the results and their implications

Using novel assays for SSCs and their differentiating progeny, we demonstrated that both self-renewal and differentiation take place in the same culture. The independence of cell fate choice with respect to the environment reveals a capacity of the rat SSC to autonomously specify its self-renewal and differentiation. Consistent with this conclusion, omitting various components from the current culture system, including feeder cells, does not eliminate the heterogeneous fate outcome (Wu et al., 2009), which suggests that none of the components of the culture

system specifically decreases self-renewal or differentiation. In addition, mathematical modeling of dynamic population growth, death, and SSC content of germ cell cultures indicates that autonomous fate decision operates according to probabilistic principles. In our hands, stochastic fate choice does not appear to be specific to rat SSCs, but also characterizes mouse SSC fate specification in culture (unpublished data). However, it remains to be examined whether the capacity for stochastic fate choice is a general attribute of most mammalian SSCs.

SSCs have been shown to display extensive parallel behaviors in culture and in vivo. Both systems support SSC self-renewal, GDNF-dependent perpetual proliferation, and a characteristic gene expression profile (Kanatsu-Shinohara et al., 2003, 2005a,b; Nagano et al., 2003; Kubota et al., 2004a,b; Hamra et al., 2005; Ryu et al., 2005; Lee et al., 2007; Oatley et al., 2007; Wu et al., 2009). Adding to the list of shared attributes, we have now shown that differentiation also occurs alongside self-renewal in both systems. Because the germ cell cultures replicate many important characteristics of SSCs in vivo, it is conceivable that stochastic fate choice may be an authentic feature of SSC behavior in vivo. However, this remains to be tested directly by experimentation in testes.

Stochastic fate choice and environmental regulation are not mutually exclusive

Maintenance of tissue homeostasis usually demands feedback regulation of stem cell activity by extrinsic physiological cues. This raises the question of how an autonomous and stochastic mechanism for SSC self-renewal and differentiation integrates input from its environment. Although our results argue against a deterministic role of extrinsic cues in SSC progeny fate specification, a nondeterministic influence of environment can fit well with the observed stochastic fate choice. For example, rather than ordaining fate of individual cells, extrinsic cues may serve to bias the probability of cell fate choices at the population level. A niche, in this case, can be viewed as an extreme environment in which fate choice is entirely skewed toward self-renewal. Therefore, stochastic fate choice and environmental regulation are not mutually exclusive but could work jointly to achieve SSC self-renewal and differentiation that is minimally dependent on the environment but responsive to its regulation.

Possible mechanisms of SSC stochastic fate choice

One possible mechanism for autonomous cell fate diversification is asymmetric cell division (Knoblich, 2008; Wu et al., 2008). In this scenario, a parent cell segregates fate determinants unequally to its daughter cells, thereby creating intrinsic differences that predestine them for different fates. However, because asymmetric division alone cannot increase stem cell number, symmetric division must also take place to produce the net increase of SSC number we observed in culture. This requires some other mechanism to account for the choice between symmetric and asymmetric divisions, which should be stochastic, for no consistent pattern of cell fate outcome was observed within SSC lineages.

Alternatively, intrinsic variation (noise) in gene expression has been shown in a few simple organisms to cause cell fate divergence within isogenic populations. Examples include entry into the competent state by *Bacillus subtilis* (Maamar and Dubnau, 2005; Smits et al., 2005; Dubnau and Losick, 2006; Süel et al., 2006, 2007) and generation of photoreceptors with alternative color vision in fly compound eyes (Wernet et al., 2006; Bell et al., 2007). These studies demonstrate that small and transient initial fluctuations in transcription of fate determinant genes can be amplified and stabilized at qualitatively different levels and cause differential cell fate outcome (Losick and Desplan, 2008). A recent study of mouse hematopoietic progenitor cells also indicates that transcription noise may control lineage choice in mammals (Chang et al., 2008). Thus, it is possible that noisy expression of genes that drive self-renewal or differentiation may play a role in fate decision of SSC daughter cells. The methodology developed in this study can be used in future investigations to identify the molecular machineries responsible for SSC stochastic fate choice.

Materials and methods

Materials and chemicals

Cell culture media, PBS nonessential amino acids, MEM vitamin solution, L-glutamine solution, 0.05% or 0.25% trypsin-EDTA solutions, B27 minus vitamin A supplement, antibiotic-antimycotic solutions, and horse serum

were obtained from Invitrogen. BSA and DMSO were obtained from EMD. Mitomycin-C, mouse laminin, mouse EGF, rat GDNF, bovine apo-transferrin, human basic fibroblast growth factor (bFGF), d-(+)-glucose, d-biotin, ascorbic acid, sodium pyruvate, putrescine, progesterone, β -estradiol 17-cypionate, insulin, sodium selenite, 2-mercaptoethanol (ME), D-lactic acid, and all fatty acids were obtained from Sigma-Aldrich. Mouse leukemia inhibitory factor/ESGRO (LIF) was obtained from Millipore. FBS was obtained from Atlanta Biologicals. Dispase and rat-tail collagen I-coated culture dishes were obtained from Thermo Fisher Scientific, Inc. The In Situ Cell Death Detection kit (TMR red) was obtained from Roche. Busulfan was obtained from MP Biomedicals. Glass-bottom dishes were obtained from MatTek Corporation.

Derivation and maintenance of rat spermatogonial cell cultures

All spermatogonial cell lines used in this study were derived from testes of 20–30-d-old homozygous male SD-Tg(ROSA-EGFP)2-4Reh Sprague Dawley rats, which exhibit germ cell-specific expression of EGFP (GCS-EGFP; Cronkhitte et al., 2005). The derivation and maintenance of the rat spermatogonial cell cultures were performed largely as described previously (Hamra et al., 2002, 2005), but without FACS-based cell sorting of EGFP-positive germ cells. In brief, seminiferous tubules were minced and digested with dispase (50 units/testis) at 32°C for up to 30 min. The dissociated testicular cells were cultured at 32°C in DHF12 medium supplemented with 5.5% horse serum, 2.5% FBS, and 1% antibiotic/antimycotic solution at 32°C in humidified incubators with 5% CO₂ in order to allow the somatic cells to attach to the plastic or gelatin-coated tissue culture dishes. After 2–3 d of incubation, the germ cells on top of the somatic cells were harvested by washing repeatedly with medium, plated on gelatin-coated dishes, and incubated at 37°C for another 2–3 h to allow remnant somatic cells to attach. At the end of the incubation, the floating germ cells were harvested and plated on laminin-coated dishes in DHF12 medium and supplemented with 10% FBS, 1% antibiotic/antimycotic solution, and 30 μ M 2-mercaptoethanol to enrich for stem cells. After 20–40 min of incubation at 32°C, germ cells that did not attach to the laminin were washed away with medium. Germ cells attached to the laminin (LamB cells) were harvested and transferred to gelatin-coated dishes in SA medium to further deplete the remnant somatic cells. After 2 d of incubation at 37°C, the floating LamB germ cells were transferred onto mitomycin-C-treated or γ -irradiated primary mouse embryonic fibroblast (MEF) feeders; then they continued to be cultured in SA medium. Routinely, the culture medium was refreshed every 3–4 d. The cultured germ cells with MEF feeder cells were passaged by trypsinization with 0.05% or 0.25% trypsin-EDTA solution onto new MEF feeders every 2–3 wk.

Single spermatogonial cell culture

To establish single cell cultures, GCS-EGFP spermatogonial cell cultures were trypsinized with 0.25% trypsin-EDTA solution to isolate and harvest the cells. After one wash, the cell pellet was resuspended at 0.5–2 \times 10⁵/ml in SA medium and filtered through 40- μ m cell strainers. 96-well plates with MEF feeder cells were washed once with PBS and replenished with 100 μ l per well of SA medium. FACS using an Aria Cell Sorter (BD) was used to deposit one EGFP-positive germ cell into each well of the 96-well plates. Medium was refreshed every 3–4 d and the growth/death of the clones was monitored using a fluorescence microscope (IX70; Olympus). The long-lasting clones were continually cultured in the original well for 2–3 mo without passaging until transplantation. Then, each clone was passaged by trypsinization with 0.25% trypsin-EDTA solution about every 2 wk.

Germ cell transplantation and analysis

Germ cell transplantations by retrograde injection through the rete of busulfan-treated wild-type rat recipient testes were performed as described previously (Ogawa et al., 1997, 1999; Hamra et al., 2002). In brief, wild-type Sprague Dawley rats at 8 d of age (Harlan Laboratories, Inc.) were injected i.p. with 12.5 mg/kg busulfan (4 mg/ml in 50% DMSO and 50% PBS) to eliminate the endogenous germ cells, then used as recipient males for germ cell transplantation at 24–29 d of age (Ogawa et al., 1999). Donor cells were harvested from long-term in vitro cultures of GCS-EGFP rat spermatogonia by 0.25% trypsin-EDTA and filtered through a 40- μ m cell strainer. EGFP-positive germ cells were counted and diluted to 3 \times 10⁴–3 \times 10⁵ cells/ml of SA or SG medium containing 0.05% trypan blue as a fluid phase marker. About 50 μ l of the cell suspension was loaded into an injection needle pulled from a 100- μ l glass capillary tube, and 20–50 μ l was then transferred into the seminiferous tubules of anesthetized rats by retrograde injection through the rete of each testis (Ogawa et al., 1997, 1999). 2 or 5 mo after transplantation, recipient testes were dissected out and spread out

on glass slides. The EGFP-positive colonies in each recipient testis were visualized and scored using a fluorescence microscope (IX70). The SSC number was then calculated based on 8% colonization efficiency (Nagano, 2003; Ogawa et al., 2003). Images of whole recipient testes were taken with a fluorescence stereomicroscope using 0.63x and 1.5x lenses (SteREO Discovery.V12; Carl Zeiss, Inc.).

Long-term time-lapse microscopy and image analysis

Cultured rat germ cells were plated at $0.5\text{--}1 \times 10^4$ cells/cm² in 25-T flasks on MEF feeder cells in SA or SG medium immediately before imaging. The flasks were mounted on the computer-controlled stage of a DeltaVision RT microscope (Applied Precision) in a temperature (37°C)- and humidity-controlled chamber. The flasks were perfused continuously with 5% CO₂. The medium was manually changed every 2–3 d without disturbing the position of the flask. Multiple fields were selected for automated time-lapse imaging. Images of each field at 4x, 10x, or 20x magnification were collected at regular time intervals of 15–30 min for 2–18 d. Time-lapse image sequences were analyzed using ImageJ (<http://rsbweb.nih.gov/ij/>). The Manual Tracking plug-in (written by F. Cordelieres, Institut Curie, France; <http://rsb.info.nih.gov/ij/plugins/track/track.html>) was used to track the centroid positions of selected individual cells.

FRAP assay for intercellular bridges and image analysis

EGFP-expressing germ cells were cultured under normal conditions in glass-bottom dishes for 4–7 d before analysis. Using a confocal microscope (LSM510; Carl Zeiss, Inc.) with a 40x/0.8 NA water immersion objective lens, photobleaching within a region of interest was performed with 488-nm illumination at 100% laser power, scanning at maximum speed 200–500 times (approximately 40 s). Time-lapse images were acquired immediately before and after photobleaching. For FRAP of EGFP germ cells in testis, seminiferous tubules were spread out between a glass slide and a coverslip. Chains of EGFP-positive germ cells near the basement membrane were selected for FRAP as described for cultured cells. The mean EGFP fluorescence intensity within a region of interest of each germ cell was measured as a function of time using ImageJ.

Mathematical models based on probabilistic fate determination

As demonstrated in the results, germ cells in culture are composed of two distinct populations: the SSCs and the differentiating germ cells. SSCs are able to proliferate indefinitely and give rise to both new SSCs and differentiating progeny. The differentiating germ cells eventually die after transient amplification, producing only differentiating cells. In addition, the invariant proportions of SSC and differentiating cells in the parent culture and in long-lasting clones derived from single SSCs (Fig. 3 A) suggest that a constant probability for self-renewal (s) and differentiation ($1 - s$) can be assumed. Therefore, the growth of the two populations over time (t) can be represented by the following two exponential equations:

$$S_t = (2s)^{t/c} S_0 \text{ and } D_t = (2s)^{t/c} S_0 (s^{-(q-1)} - 1),$$

where S_t and S_0 are the number of SSCs at time t and time zero, respectively, D_t is the number of differentiating germ cells at time t , and q denotes the number of divisions required to produce the average number of progeny among the differentiating cells before they die. In both equations, c is the mean cell cycle length. Measurements from time-lapse imaging showed that c is the same for SSCs and differentiating cells (Fig. 5 A).

Therefore, at time point t , the total germ cell number (G_t) is:

$$G_t = S_t + D_t = (2s)^{t/c} S_0 (s^{-(q-1)}).$$

Taking the natural log of the above equation, we obtained:

$$\ln G_t = t/c \ln (2s) + \ln (S_0 s^{-(q-1)})$$

When $t = 0$, then $G_0 = S_0 (s^{-(q-1)})$. Therefore, the equation above becomes:

$$\ln G_t = [\ln (2s) / c] t + \ln G_0. \quad (1)$$

Experimental values for G_0 and G_t were measured as the total number of germ cells at the beginning and at subsequent time points. A linear least squares fit to a semilog plot of the measured data ($\ln G_t$ vs. t) has a

slope of 0.2064 ± 0.002245 . Hence, Eq. 1 best fits the measured growth kinetics (Fig. 5 B) when $\ln (2s)/c = 0.2064$. As the measured average cell cycle length $c = 1.67$ d (40 h), $s = 0.706$.

As experimentally determined, differentiating cells have limited proliferation capacity, assuming a differentiating cell can divide on an average q number of divisions before it dies, then in a given germ cell group of total number G_0 with S_0 number of SSCs, the derivative of the percentage of live clone (P_{alive}) derived from this population at a given time point t is

$$dP_{alive} / dt = -S_0 \times (1-s) \times s^{-(q+1)+t/c} / G_0, \text{ when } t < (q+1)c_{max}.$$

Therefore, P_{alive} is an exponential decay function of t , when $t < (q+1)c_{max}$:

$$P_{alive} = -c S_0 (1-s) s^{-(q+1)} / [G_0 \ln (s)] s^{t/c} + S_0 / G_0. \quad (2)$$

Because exponential decay function $P = (P_0 - \text{Plateau}) e^{-(K)t} + \text{Plateau}$ best fit to the measured data ($R^2 = 0.9720$) when $K = 0.2886$, $P_0 = 100\%$, and $\text{Plateau} = 20.02\%$, our model best fit with the measured data (Fig. 5 C) when $s = 0.618$, $S_0/G_0 = 20.02\%$, and $q = 3.3$.

Plugging in the exponential expressions for S_t and D_t , the stem cell fraction (R_s) is:

$$R_s = S_t / G_t = s^{(q+1)}. \quad (3)$$

Because R_s is not a function of time, this model predicts that the stem cell ratio remains unchanged over time. Assuming $s = 0.67$ and $q = 3.3$, it predicts that the stem cell ratio in culture should remain constant, with a value of 17.9%.

Online supplemental material

Fig. S1 shows that a mortal population persists in long-term rat germ cell cultures. Fig. S2 shows flow cytometric analysis of cultured GCS-EGFP rat germ cells for c-kit expression. Table S1 shows that mortal germ cells in culture die synchronously as sibling groups. Video 1 shows synchronized cell division and death in a clone of germ cells. Video 2 shows EGFP exchange between cultured germ cells through intercellular bridges. Video 3 shows that daughter cells of a single SSC dynamically sample the same microenvironment.

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