

Stem Cells Are Units of Natural Selection in a Colonial Ascidian

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SUMMARY

Stem cells are highly conserved biological units of development and regeneration. Here we formally demonstrate that stem cell lineages are also legitimate units of natural selection. In a colonial ascidian, *Botryllus schlosseri*, vascular fusion between genetically distinct individuals results in cellular parasitism of somatic tissues, gametes, or both. We show that genetic hierarchies of somatic and gametic parasitism following fusion can be replicated by transplanting cells between colonies. We prospectively isolate a population of multipotent, self-renewing stem cells that retain their competitive phenotype upon transplantation. Their single-cell contribution to either somatic or germline fates, but not to both, is consistent with separate lineages of somatic and germline stem cells or pluripotent stem cells that differentiate according to the niche in which they land. Since fusion is restricted to individuals that share a fusion/histocompatibility allele, these data suggest that histocompatibility genes in *Botryllus* evolved to protect the body from parasitic stem cells usurping asexual or sexual inheritance.

INTRODUCTION

Generation of tissues and organs during development is carried out by stem cells, which are uniquely endowed with both the capacity for self-renewal and the potential to give rise to differentiated cells. In vertebrate embryos, transitory embryonic stem cells (ESC) generate all three tissue layers, and in mammals, ESC can contribute to the entire organism

when injected into blastocysts (Gardner and Rossant, 1979; Martin, 1981). Stem cells with restricted potential persist throughout adult life for the repair and regeneration of specific organs such as the skin, brain, and blood (for review, see Weissman, 2000). By contrast, entire bodies are regenerated many times throughout life in colonial organisms found in several phyla (for example, Lauzon et al., 2002). It has been hypothesized, though not shown, that development of asexually derived bodies in colonial organisms proceeds from a pool of stem cells not unlike ESC (Stoner et al., 1999). Such stem cells for asexual regeneration would be required throughout the life span of the organism since development occurs many times over.

An intriguing corollary question concerns the origin of gametes in clonally replicating organisms. As in vertebrates, roundworms, and flies, the cell lineage destined to become the germline may be segregated very early in embryogenesis; this scenario would require the maintenance of separate lineages of germline stem cells and somatic stem cells throughout all asexual generations. Alternatively, a single pluripotent stem cell lineage spawns both bodies and gametes, perhaps generating downstream pools of somatic and germline stem cells or progenitors in the process. In either case, the continued derivation of many generations of organisms from a small pool of stem cells places an evolutionary premium on those cells. The lineage that propagates the genome by homing and infiltrating the site of gametogenesis—whether it is a germline stem cell or pluripotent stem cell—is an irreducible unit of natural selection (Stoner et al., 1999; Weissman, 2000).

We have undertaken a prospective isolation of the stem cells that regenerate bodies in the ascidian *Botryllus schlosseri*. Colonies are composed of clonogenic individuals, termed zooids, sheathed in a gelatinous tunic and connected to each other by an extracorporeal vascular network. Asexual reproduction occurs by outgrowth of one to three buds from the zooid wall (Berrill, 1941; Izzard, 1973) in a highly coordinated weekly cycle that is punctuated by the death and resorption of the entire generation of mature zooids, coincident with the succession of the next generation (termed takeover; Lauzon et al., 1992). Colonies attain sexual maturity at approximately 3 months of age, marked by the intermittent generation of testes and ovaries (De Tomaso

et al., 1998; De Tomaso and Weissman, 2003; Milkman, 1967; Mukai and Watanabe, 1976). Tadpole larvae disperse by swimming and undergo metamorphosis to a single zooid following attachment to a vertical substrate (Milkman, 1967).

Botryllus colonies vying for resources in subtidal niches impinge upon one another at their borders (Grosberg and Quinn, 1986). There, terminal vascular structures called ampullae make contact, which results in an allorecognition event with two possible outcomes determined by a highly polymorphic histocompatibility locus called the FuHC (for fusion/histocompatibility, see De Tomaso et al., 1998, 2005; De Tomaso and Weissman, 2003; Scofield et al., 1982). An inflammatory reaction ceases interaction between colonies lacking a common FuHC allele (Oka and Watanabe, 1957). By contrast, colonies sharing one or both FuHC alleles undergo vascular fusion and the establishment of a common circulation (Scofield et al., 1982). Following this parabiosis, or fusion, of two genetically distinct colonies, the asexual progeny and gametes of one or both partners become a blend of both genotypes or, in some cases, are completely replaced by the cells of the other (Stoner and Weissman, 1996). This chimerism persists long after severing blood vessels connecting the fused colonies, and it follows genetically heritable hierarchies for germline “winners” and “losers” (Stoner et al., 1999). The reproducibility and longevity of this phenomenon suggested to us that the causative agents were blood-borne stem cells—either separate lineages of somatic and germline stem cells or, alternatively, a single pluripotent stem cell population with differing somatic and gametic competitive strengths. Here we describe the use of *Botryllus* as an experimental system to isolate the cell lineage(s) responsible for parasitic hierarchies. Our criteria for a candidate stem cell were both the induction of long-term, stable, multilineage chimerism and demonstrated self-renewal potential (Abramson et al., 1977; Becker et al., 1963; Till and McCulloch, 1961; Weissman, 2000; Wu et al., 1968). We show by limiting dilution transplantation that a highly enriched population of cells contributes to either somatic or germline fates, though not both, and that engraftment ability of these cells reproduces parasitic hierarchies of natural colony fusion.

RESULTS

The unique biology of *Botryllus* facilitated the development of a transplant assay between genetically distinct but FuHC-compatible colonies without requiring radiation-induced death of host stem cells (Laird and Weissman, 2004a; Till and McCulloch, 1961). Cells can be successfully delivered by ampullar microinjection (Figure 1) and detected circulating in sites throughout the vasculature and zooids after 24 hr (Figures S1B and S1C). To determine the frequency of cells inducing genetic chimerism in parabiosed partners, cell suspensions were prepared from one system of a *Botryllus* colony (a rosette of 3–12 zooids with a common atrial siphon and shared extracorporeal vasculature), and a test number of cells was transplanted into a histocompatible recipient system by ampullar microinjection. Somatic tissues and

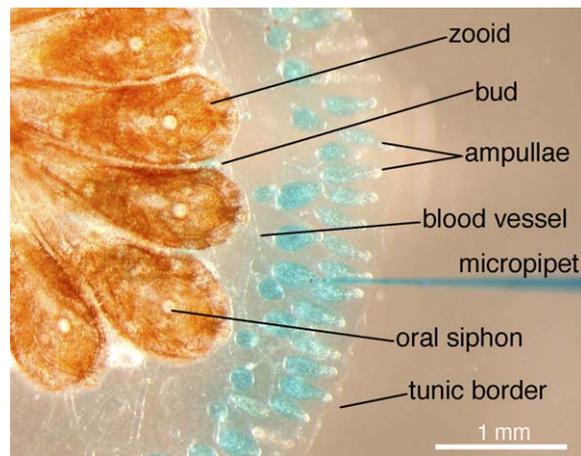


Figure 1. Anatomy and Cell Transplantation in *Botryllus schlosseri*

Microinjection of isosulfan blue dye into the ampulla of a colony results in labeling of blood vasculature, asexual buds, and zooids. Abbreviations: amp, ampulla; bv, blood vessel.

testes were harvested after 1–3 months, and chimerism was assayed by allele-specific PCR markers in the FuHC locus (Figure S1A and Table S1; De Tomaso et al., 2005).

Establishing Stem Cell Frequency by Limit Dilution Transplantation

We predicted that *Botryllus* stem cells would be blood-borne since (1) vascular contact appears to be sufficient for genetic parasitism in fused colonies (Oka and Watanabe, 1957) and (2) regeneration of the entire organism from the blood vasculature has been reported in *Botryllus* (Oka and Watanabe, 1959b; Voskoboynik and Weissman, personal communication) and an ascidian of the same genus (Oka and Watanabe, 1959a). However, we found that somatic cell engraftment required higher doses of donor blood cells than total cell suspensions (Table 1); a reduced frequency of chimera-forming activity in the blood as compared with the entire body implies that stem cells do not reside primarily in the blood. For donor genotypes capable of winning in a fusion (competent donors), the limiting dose for somatic chimerism was 500–1000 cells from an entire system; however, the data suggest that over 5000 blood cells would be required to produce a 63% engraftment rate which, by statistical prediction, is achieved at the dose containing an average of one stem cell (Henry et al., 1980). Somatic and, where examined, germline chimera formation occurred at similar frequencies when transplants came from competent donors. These results imply that the stem cells that parasitize somatic tissues reside and proliferate in a sequestered compartment and migrate via blood circulation to seed the forming buds and gonads. Much like hematopoietic stem cells in mammalian bone marrow (Wright et al., 2001), the residence time of these primitive cell populations in the blood is short. A limiting dilution of 500–1000 cells from a donor colony implies that the frequency of stem cells populating the somatic and gonadal niches ranges between 1:500 and 1:1000 in the entire

Table 1. Limiting Dilution Transplantation of Winner and Loser Genotypes

Cell Population	# Cells Transplanted	Somatic		Germline		Soma + Germ	
		n	%	n	%	n	%
Competent Donor							
Total cells	10,000–20,000	4	100	—	—	—	—
	5,000	1	100	—	—	—	—
	2,000	5	80	4	75	4	75
	1,000	5	60	—	—	—	—
	500	8	63	10	60	7	57
Blood	4,000–5,000	2	50	—	—	—	—
	1,000–2,000	4	25	—	—	—	—
Incompetent Donors							
Total cells	10,000	1	0	—	—	—	—
	2,000	3	0	1	0	—	—
	1,000	2	0	—	—	—	—
	500	6	0	1	0	—	—
Blood	2,500	3	0	—	—	—	—

Somatic and germline tissues (if they arose) were analyzed for the presence of donor genetic markers 1–3 months following transplant with indicated numbers of blood or unfractionated cells from a donor that can win in a fusion (competent) or a donor that loses during fusion (incompetent). The number of different transplants and percentage of those resulting in detectable chimeras are indicated for somatic outcomes, germline outcomes and, where donors were competent in somatic and germline, coincident contribution to both outcomes; a dash indicates the absence of data. For all sets of donor and host genotypes, a dilution curve of each naïve DNA indicated the sensitivity of each particular polymorphism. The limit of detection of donor DNA in the host varied from as little as 1:50 to more than 1:10.

body. However, these results do not distinguish between the possibilities of one pluripotent stem cell with differing competitive fitnesses in the soma and germline or, alternatively, two separate lineages of stem cells that exist at similar frequencies in the body.

When genotypes from colonies that consistently lose in a test fusion were transplant donors, we found that allogeneic cell engraftment replicates the hierarchies of genetic parasitism observed in vascular fusion of the same colony genotypes. “Loser strain” donor contribution to somatic or germ tissues was never detected in recipients, even at doses greater than 10-fold over the previously established limiting dilution of 500–1000 (Table 1). In reciprocal transplants between FuHC-compatible pairs, outcomes usually corroborated parasitic hierarchies observed in test fusions with subclones of the same genotype (Table 2). In cases of well-matched competitors that mutually or variably parasitized one another in a fusion, such as the germline genotypes m and w (Table 2, line 3), correlation was not absolute. In other cases, insufficient numbers of transplanted cells likely ac-

Table 2. Hierarchies in Natural Fusion versus Transplantation

Genotypes Tested	Fusion Tissue	Fusion Winner	Transplant Corroboration		
			Total Cells	BAAA ⁺ Cells	Total Transplants
C vs. w	SOMA	w	5 / 5	6 / 7	11 / 12
	GERM	c ≥ w	3 / 4	4 / 7	7 / 11
W vs. m	SOMA	w > m	4 / 4	—	4 / 4
	GERM	m > w	1 / 3	—	1 / 3
A vs. b	SOMA	a > b	6 / 7	—	6 / 7
	GERM	b > a	7 / 7	—	7 / 7

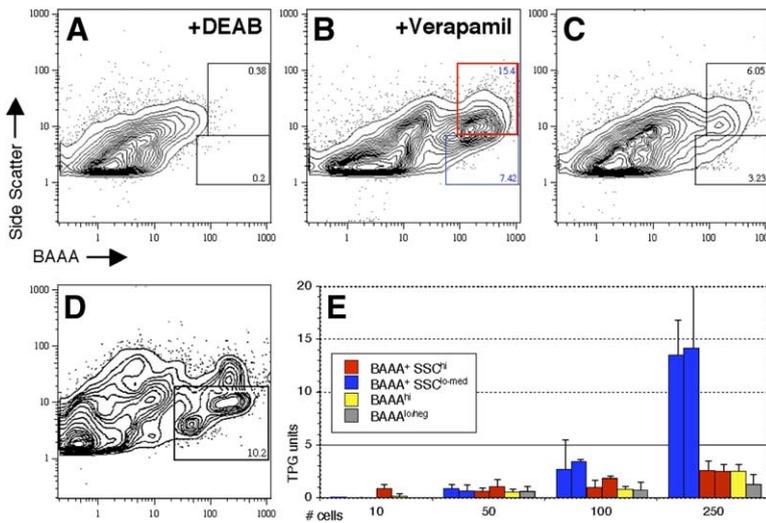
Somatic and germline outcomes were compared following fusion, reciprocal transplant of 500 or 2000 total cells, or 50–150 BAAA⁺ cells. The winner in a test fusion is represented as the genotypes detected in the resulting parabiont; ≤ or ≥ is used for parabionts in which an equal or near-equal ratio of genotypes was detected. The number of cases in which the transplant result corroborates the winner of fusion is indicated.

count for discrepancies between parasitic hierarchies and transplant outcome. However, gametic or somatic engraftment at limit dilution was extremely high when donor genotypes were “winners” that repeatedly and absolutely parasitize fused partners. This correspondence and the failure of a large number of loser-to-winner transplants to engraft indicate that loser phenotypes are inherent to these cells.

Prospective Enrichment of Stem Cells

Enrichment of stem cells was carried out with a viable cell-permeant fluorescent aldehyde dehydrogenase (ALDH) substrate, bodipy aminoacetylaldehyde (BAAA), which delineates hematopoietic stem cells (HSC) and progenitors in humans and mice (Fallon et al., 2003; Jones et al., 1995; Storms et al., 1999). ALDH is present both in mammalian HSC (Jones et al., 1996) and, suggestively, in the growing asexual buds of another colonial ascidian, *P. misakiensis* (Hara et al., 1992; Kawamura and Fujiwara, 1994, 1995). By flow cytometry, BAAA⁺ cells compose 10%–20% of *Botryllus* total cell suspensions; this population decreases in most cases in the absence of the efflux inhibitor verapamil and disappears in the presence of the ALDH inhibitor DEAB (Figures 2A–2D). Since the flow cytometric profile and behavior of *Botryllus* cells in BAAA appeared similar to mammalian bone marrow, we proceeded to clone sort BAAA⁺ cells as well as side scatter (SSC) high and low subpopulations.

Functional engraftment capacity of each BAAA cell fraction was tested by transplantation. Somatic chimerism, germline chimerism, or both could be produced in FuHC-compatible recipients with 200 or as few as 25 BAAA⁺ cells. The results from transplantation of BAAA⁺, BAAA⁺ SSC^{hi}, and BAAA⁺ SSC^{lo} cell populations were statistically indistinguishable and are therefore combined (Table 3). Genotyping of individual organs and testes at various time points revealed an increase in detectable chimerism between 1 and



3 months following transplant. The limit dilution for somatic tissue contribution is approximately 25–50 BAAA⁺ cells and appears to be slightly higher for germline chimerism and coincident somatic/germ tissue engraftment. All together, this signifies a greater than 10-fold enrichment for chimera-forming activity in the BAAA⁺ population over unfractionated cells. The low frequency of BAAA⁺ cells relative to the entire population (10%–20%, gating-dependent) suggests that stem and progenitor cells do not exist in significant amounts in the BAAA^{lo/neg} population. As expected, transplantation of 25 BAAA^{lo/neg} cells to compatible recipients did not produce detectable somatic or germline contribution after 2 or 4 months (data not shown).

Assaying Multilineage Contribution and Self-Renewal

The capacity for multilineage contribution and self-renewal, the hallmark of stem cells, was assayed in the BAAA⁺ population using several different experimental approaches. First, pigment cell differentiation was used to assess somatic dif-

ferentiation of transplanted cells. In these cases, we observed an accumulation of donor-type pigment cells in the ampullae and zooid mantle in addition to donor allelic markers in these tissues (Figures 3A–3C). The derivation of most or all donor-type pigment cells from transplanted stem or progenitor cells is likely because pigment cells are, for the most part, lysed during flow cytometry due to their size and fragility and are not detected by cytosin analysis of BAAA⁺ cells (data not shown). Therefore, despite extremely slow turnover (Lauzon et al., 2002), the appearance of donor pigment cells in a recipient of limiting numbers of BAAA⁺ cells implies de novo generation from the transplanted population and serves as a phenotypic marker of somatic tissue chimerism. Next, in selected instances of germline chimerism, functional replacement of host colony gametes was confirmed by test cross of germline chimeras 3 months following transplant. Using recessive blue pigmentation as a phenotypic marker, an orange (heterozygous) recipient of blue BAAA⁺ cells crossed to a blue-pigmented mother produced only 7/110 (6.3%) orange progeny; the deviation of this result from the expected 50% Mendelian ratio confirms the germ cell parasitism detected by genotyping. Finally, spatial distribution and expansion of the BAAA⁺ population was examined in several recipient colonies by selective sampling of visceral organs and testes from different locations over time. In one representative experiment, donor genotype somatic and germline contribution could be detected in peripheral zooids of a colony growing from a single system at transplantation, to six systems at 1 month and up to 41 systems after 3 months (Figure 3D–3F). Somatic and gametic chimerism did not always coincide spatially, but the wide distribution of donor-derived cells at few randomly selected locations throughout the colony over 3 months implies the continued and prolific expansion of cells among the original 50 BAAA⁺ cells injected at transplant.

In order to test the self-renewal potential of the BAAA⁺ population more definitively, we performed serial transplantations. BAAA⁺ cells were reciprocally transplanted between FuHC-compatible colonies h and e that exhibited

Table 3. Transplantation of BAAA⁺ Cells

# Cells Transplanted	1 Month		3 Months							
	SOMA		SOMA		GERM		BOTH			
	n	%	n	%	n	%	n	%		
200	2	50	1	0	2	100	1	100	1	100
100	9	78	5	80	1	100	1	100	1	100
50	7	86	2	100	7	71	6	50	5	40
25	5	40	2	100	5	60	4	25	4	25

Data shown include both BAAA⁺ SSC^{hi} and SSC^{lo} subpopulations as no functional difference between these subpopulations could be discerned. For each cell dose, we report percentage of recipients in which donor contribution was detected in somatic tissues, germ tissue, or (where eligible) both at 1 month or 3 months following transplant. Differing n reflect the absence of gonads in recipient colonies during the observation period.

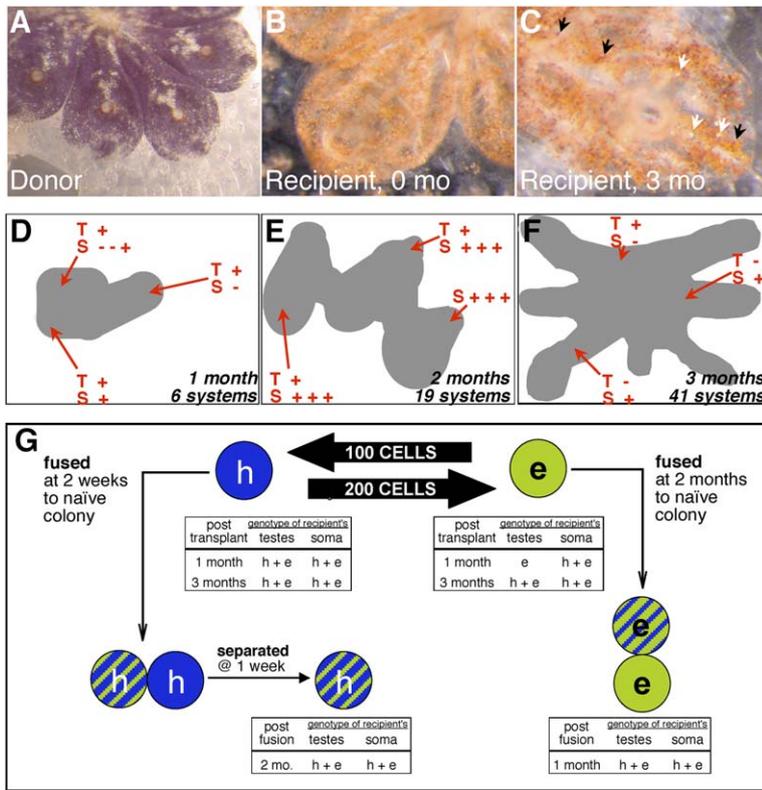


Figure 3. BAAA⁺ Cells Are Capable of Multilineage Differentiation and Self-Renewal

(A–C) Transplanted cells expand and contribute to recipient pigmentation. Fifty BAAA⁺SSC^{hi} cells from a blue, orange, and white pigmented donor (A) were transplanted to an FuHC-compatible orange recipient (B); 3 months following transplant, a mixture of white (white arrows), blue (black arrows), and orange cells are dispersed throughout the zooid mantle of the recipient (C).

Multilineage contribution was assayed by genotyping multiple organs (including the testes, intestine, endostyle, mantle, and branchial basket) from different locations in colonies receiving 50 BAAA⁺SSC^{hi} cells after 1 month (D), 2 months (E), and 3 months (F). Diagrams represent colony size and shape as well as positions of the testes (T) and somatic organs (S) from a single zooid (arrows); the presence or absence of donor-marker detection in each tissue are represented by a plus (+) or minus (–). The size of the colony at each time point is indicated by the number of systems. (G) Serial transplantation was used to assay self-renewal. Recipient e of 200 BAAA⁺ h cells was fused to another colony of genotype e 2 months following transplant (right), and 1 month later genotype h could be detected in that fusion partner (see boxes), as indicated. Recipient h (top) of 100 BAAA⁺ genotype e cells was fused to another h colony 2 weeks following fusion (left); this pair was surgically separated after 1 week, and 2 months later the original donor markers could be detected in the second recipient.

bidirectional somatic and germline parasitic potential in a fusion and were thus equal competitors. Recipient e (Figure 3G) was fused to a naïve syngeneic subclone 2 months following transplant; 1 month later, h donor-derived markers were detectable in the fused e parabiont. It would be expected that if the originally transplanted population contained only progenitor cells, they would have expired after 3 months. Similarly, 2 weeks following transplant, recipient h was temporarily fused to a naïve syngeneic colony for 1 week; 2 months later, cells from the original donor, e, were present in both somatic and germline tissue of the surgically separated fusion partner. Both permutations of this serial transplant experiment demonstrate the self-renewal as well

as multilineage differentiation capacity of BAAA⁺ cells upon transplantation.

Can Transplantation Circumvent Histocompatibility Barriers?

We next used this transplantation system to ask whether all-recognition of colonies also results in all-rejection of transplantable cells. Total cell suspensions transplanted 10-fold over limit dilution were not capable of inducing detectable chimerism in somatic organs or testes of FuHC-incompatible recipients by allelic marker detection or pigment cell presence (Table 4). Since transplantation bypasses the process of fusion, this result supports the hypothesis that the FuHC

Table 4. Results of Transplantation across Fu/HC Barriers

Donor Cell Population	# Cells Transpl.	Donor Marker Detection in Recipient Tissues						
		System	Intestine	Br. Bas	Endost.	Buds	Testes	Pigment Change
Total	10,000	0/1	—	—	—	—	—	no
	8,000	0/1	0/1	—	—	0/1	0/1	no
	8,000	—	—	—	—	0/1	0/1	no
BAAA ⁺	400	-	0/2	0/1	0/1	-	-	no
	250	-	0/2	0/1	0/2	-	-	no

Tissues surveyed include entire systems, intestine, brachial basket, endostyle, buds, and testes. Donor and host were different pigment types in all cases.

in *Botryllus* depends on cellular allorecognition rather than an anatomic barrier created at the site of rejection. To test the possibility that long-lived self-renewing stem cells do not express targets of allorecognition, we transplanted 250 or 400 BAAA⁺ cells between FuHC-incompatible colonies. Donor allelic markers could not be detected in host tissue after 6 weeks or 3 months. It is therefore likely that stem cells or their downstream progenitors harbor allorecognition molecules that induce their destruction or prevent their infiltration into sites of somatic or germline engraftment.

Separation of Somatic and Germline Outcomes

Since the statistical outcomes for somatic and germline chimerism do not clearly separate for unfractionated or BAAA⁺ cell recipients in the aforementioned experiments, we developed limit dilution or “single”-cell transplantation methodology to attempt to determine whether the BAAA⁺ population is composed of a single pluripotent lineage or both somatic and germline stem cells. Single-cell contribution to both lineages would demonstrate pluripotency, but single cell contribution to somatic or germline, not both, could be interpreted either as separate stem cells or as pluripotent cells that commit to one or the other fate in a microenvironment-determined manner.

We had observed some variability in the SSC profile of the BAAA⁺ population, most notably the appearance in some preparations of distinct SSC peaks in the FACS profile (Figure 2D). Clone sorting of a more liberal SSC^{lo-med} subset of the BAAA⁺ cells revealed a significant elevation in telomerase activity over other BAAA populations (Figure 2E). As increased telomerase activity in stem and immortal cells in other systems reflects an increased capacity for self-renewal (Morrison et al., 1996), we elected to use this BAAA⁺ SSC^{lo-med} population for single-cell transplants. Due to the technical constraints of the system, cell number was determined volumetrically by dilution of the test population with carrier cells of the recipient genotype. Injection of a precise volume of cell mixture corresponded to transplantation of between 0 and 5 donor BAAA⁺ SSC^{lo-med} cells, as described by the Poisson distribution (Table S2). As expected from results of BAAA⁺ and total cell preparations, single-cell transplants from poor somatic or germline donors did not produce chimerism in recipients (Table 5). However, when donor cells came from genotypes capable of winning in a fusion, we were able to detect germline chimerism in 3 out of 10 cases and somatic chimerism in 4 out of 15 cases after 2–4 months. Visual corroboration of the latter result was furnished by the appearance of donor pigment-type cells in the zooid mantle of two recipients with the highest level of engraftment in multiple somatic organs (1342 XI and 1342 XII). In two other cases, a few donor-type pigmented cells were observed in the mantle of recipients for which donor contribution could not be detected by allelic markers, possibly because donor cells were too few. Though low levels of chimerism could be detected at a single time point in intestines of one germline chimera (1342 IX) and in the testes of one somatic chimera (1342 XI), we did not observe long-term, stable, or substantial contribution to both lineages in

any case. Given the heterogeneity of the transplanted cell population, the success rate of these experiments indicates a highly efficient homing system of the engrafting cells. The failure to observe robust, coincident somatic and germline chimerism in any single-cell recipients is most consistent with the argument that separate lineages of somatic stem cells and germline stem cells exist in *Botryllus*. Similar frequencies of somatic and gametic tissue engraftment in all of the experiments described here suggest that both stem cell pools, if separate, are maintained at similar numbers in the body. It is possible that the engrafting cell population injected is in fact pluripotent but in each case differentiated to either somatic or germ lineages, depending on microenvironment signals received after transplantation. The issue can only be resolved by the isolation to homogeneity of each of these separate stem cell populations, presumably using markers that subdivide the ALDH⁺ pool, followed by transplantation of doses much larger than 25 cells per host.

DISCUSSION

In the evolution of development, the properties of self-renewal and multipotency have been restricted to stem cells. During embryogenesis, stem cells with broad or unlimited potential function as the building blocks of organisms, but only specialized stem cells survive for the maintenance of specific organs during their comparatively long lifespan. An organism that propagates itself asexually through many generations of short-lived bodies in theory harbors one or more lineages of stem cells capable of entire body regeneration—an adult version of ESC. We have taken a prospective approach to isolate a population of stem cells in the colonial ascidian *Botryllus schlosseri*. We exploit a natural histocompatibility reaction in this species to identify an enriched population of cells that, when transplanted to fusible but genetically distinct recipients, produces either somatic or gametic chimerism. Engraftment of cells followed a dose-response curve and replicated genetically determined parasitic hierarchies associated with vascular fusion of colonies. The self-renewal and separate somatic or gametic potential of this population leads us to believe that it contains lineages of somatic stem cells and germline stem cells; these lineages compete with the stem cells of closely related individuals for access to niches of nascent gonads or blastogenic buds that will form the bodies of the next generation.

Stem Cells as Units of Natural Selection

As the effectors of repeated development of entire bodies and germlines rather than repair or regeneration of specific organs, stem cells in *Botryllus* are irreducible units of evolution. In this paradigm, it is the robustness of stem cells in addition to the robustness of bodies that transmit the genome to the next generation (Laird and Weissman, 2004b). As units of natural selection, *Botryllus* stem cell lineages that generate bodies are themselves under pressure to proliferate and successfully populate niches of forming buds within isogenic colonies. An additional level of complexity arises in colonies that have fused with one or more other partners.

Table 5. Single-Cell Transplant of BAAA⁺ SSC^{lo-med} Cells

Recipient Genotype	1–2 months				3–4 months				Pigment Change?
	Br Bas/ Mantle	Endostyle	Intestine	Testes	Br Bas/ Mantle	Endostyle	Intestine	Testes	
Competent Donors									
1342 I	○	○		○					no
1342 III	○		○	●					no
1342 IV			○	●					no
1342 VI	○	○	○	np			○	np	no
1342 VII			○	np			○	np	no
1342 IX	○		○	np			○	●	no
1342 X	○	○	○	○	○	○		○	weak
1342 XI	●	●	○	○	●	○	○	○	yes
1342 XII	○	●	○	○	●	●	●	○	yes
1296p I	○	○	○	○					no
1296p III			○	np	○	○	○	np	weak
1296p IV	○	○	○	○					no
1296w I					○	○	○	np	no
1296w II	○	○	○	np	○	○	○	np	no
Hm2e IV			●	○					n/a
Hm2e V					●	●		np	n/a
Incompetent Donors									
Hm2a I					○	○		np	n/a
Hm2a II					○	○	○	np	n/a
Hm2a III					○	○	○	○	n/a
Hm2a IV					○	○		np	n/a
Hm2a V					○	○	○	np	n/a
Totals									
			Competent Donor		Incompetent Donor				
Somatic chimeras			4 / 15 (26%)		0 / 5				
Germline chimeras			3 / 10 (33%)		0 / 1				

Roman numerals in the genotype shown denote subclones of the same genotype, and letters indicate siblings. Colony 1342 subclones received transplants from two different progeny, and colony pairs 1296p and 1296w and Hm2a and Hm2e were reciprocally transplanted. The absence (○) or presence (●) of donor genotype markers in each recipient tissue is indicated with unfilled or filled circles, with weak or tentative presence indicated by gray circles; np = not present; n/a = not applicable (i.e., donor and host were the same pigment type).

Here, the cellular phenotype becomes more important for selection, as stem cell lineages in chimeras directly compete for access to the sites of blastogenesis and gamete formation. We imagine that the fitness criteria include properties common to stem cells in many organisms, such as proliferation, survival, and homing (Stoner et al., 1999).

The mechanism by which selection acts on stem cells in *Botryllus* depends upon whether separation of somatic and germline lineages is maintained throughout life. Somatic stem cells capable of winning in a fusion will not be selected

in a chimera unless they share fitness genes with the germline stem cells they nurture. Conversely, superpredatory germline stem cells can only survive to gametogenesis in fit bodies, so evolution would favor the subset of successful germline stem cells that share body fitness genes. If soma and germline are derived from a pluripotent stem cell, then fitness genes for both competitions inhere in the same stem cell subset.

The capacity for sequential or simultaneous fusion with many adjacent colonies enables colonies to collect many pools of stem cells. The advantages for retaining multiple

genomes, as has been documented in the wild (Chadwick-Furman and Weissman, 2003; Paz and Rinkevich, 2002), potentially enable a colony to respond to changing ecological conditions by changing its somatic or gametic composition as well as optimizing for the best genes for robust bodies or productive germline. At a genetic level, such a chimera can test new combinations of genes long before that particular set could be gathered into a single genome by chromosomal reassortment and meiotic recombination.

A Stem Cell Isolatable by Phenotype

It is significant that parasitic hierarchies manifested between fused colonies can be replicated by transplantation of even a single cell between those genotypes. These experiments demonstrate that competitive properties do not arise from nonautonomous signals or mechanisms but are inherent to the stem cells themselves. In other model systems, competitive phenotypes of cells based on gene-expression levels have been observed (de la Cova et al., 2004) or induced by genetic modification (Domen et al., 2000). However, in *Botryllus*, the genetic heritability of these traits for at least germline parasitism (Stoner and Weissman, 1996) indicates that they have evolved by natural selection and that their genetic loci can be identified and cloned. The next steps will be to generate reagents such as monoclonal antibodies for the resolution of homogenous somatic stem cell and germline stem cell populations as well as the identification of cell subsets that express FuHC antigens and homing receptors. Characterizing the location and the nature of the somatic and gametic niches that become the objects of intense competition between stem cell lineages is an important avenue for the future.

Stem Cell Parasitism Regulated by Histocompatibility Genes

Unlike prospective isolation approaches in mammals, donor-cell engraftment induced by cell transplant in *Botryllus* reproduces naturally occurring phenomena. On both a cellular and anatomical level, parasitism is restricted to colonies capable of vascular fusion. The genetic locus that governs fusion of colonies therefore also functions as the gatekeeper to invasion by stem cells. This locus, the *Botryllus* FuHC, was very recently characterized and encodes a class of highly polymorphic and variable genes unrelated to mammalian major histocompatibility complex genes (De Tomaso et al., 2005). This new molecular evidence complements observations of hundreds of alleles in wild *Botryllus* populations based on fusion capability (Rinkevich and Weissman, 1991; Scofield et al., 1982). The degree of polymorphism at the FuHC locus within populations diminishes the likelihood of shared alleles between unrelated colonies. Fusion and the accompanied potential for stem-cell parasitism are thus restricted to kin. In the absence of a histocompatibility locus, we imagine that the potential for fusion between all individuals would result in the metastasis of "supercompetitor" stem cell genotypes that would intercept Mendelian inheritance and homogenize entire populations. Following FuHC-permitted fusion of individuals sharing at least one allele, although ga-

metric parasitism forfeits an individual's opportunity to propagate its own genome, 25%–50% of that genome can still be propagated if the fusion partner is a sibling or parent.

The demonstration here that FuHC genes regulate the parasitism by stem cells suggests the coevolution of these two phenomena in *Botryllus*. The prevention of unchecked invasion of germline or soma by predatory stem cells, in other words, might act as a driver of polymorphism at the FuHC locus (Buss, 1982; Buss and Green, 1985; Stoner et al., 1999; Stoner and Weissman, 1996). Previous work proposes a mechanism for *how* FuHC diversity is maintained by reduced fitness of juvenile homozygotes (De Tomaso and Weissman, 2004). However, the question of *why* such extraordinary polymorphism is maintained remained speculative. If, as we have shown here, predatory stem cells are the effectors of tissue parasitism between fused colonies, then the FuHC locus effectively serves as a metric of genome similarity between colonies and even an enabler of kin selection (Hamilton, 1964).

CONCLUSION

Stem cell competition and gametic parasitism, as we have described in a colonial ascidian, may be mechanisms for genome propagation in many organisms. A very recent discovery of a putative germline stem cell population in mouse bone marrow (Johnson et al., 2005) suggests that stem cells in vascular circulation generate oocytes throughout adult life, much like blood-borne germline stem cells in *Botryllus*. If germline stem cells can be prospectively isolated in mammals and are capable of crossing the placenta, as is documented for hematopoietic stem cells (Bianchi et al., 1996; O'Donoghue et al., 2004), we hypothesized that the potential could arise for parasitism of the maternal germline by the fetus (and father of that fetus). Stem cells of the germline in mammals and colonial ascidians might share migratory programs, and a mechanism for regulating such parasitism could exist in mammals. For *Botryllus*, more profound understanding of the coevolution of histocompatibility and stem cell parasitism may come from the characterization of the FuHC locus as well as the genetic elements that determine stem cell hierarchies.

EXPERIMENTAL PROCEDURES

Animals

Botryllus colonies used for transplant experiments included lab-cultivated strains and oozoids spawned from colonies collected around Monterey Bay and maricultured as previously described (Boyd and Weissman, 1986). Subclones were tested for fusibility with their siblings by cut-colony assay (Rinkevich and Weissman, 1987). Recipient colonies were transferred as single systems onto separate slides at least 1 week prior to transplant to permit firm attachment. For cases in which oozoids were paired with their mothers as donor or recipient, it was not possible to perform the cut-colony assay. Oozoids were allowed to become a colony consisting of at least two zooids before one zooid was removed for DNA extraction. Tissue harvesting for genotyping was performed with a Wheeler dissecting knife (Ernest Fullam, Inc.) under stereomicroscopy. Following transplant, colonies were cleaned and observed at least once per week to assess their health and remove from the slide newly settled oozoids that could potentially serve as a source of contaminating DNA

by fusion. One to four months following transplant, testes and somatic tissues including the intestine, branchial basket, endostyle, and mantle were surgically excised from the colony during midcycle for genotyping.

Cell Staining

Colonies to be analyzed by flow cytometry were rinsed in sterile seawater and sliced open with a razor blade. Cells were dissociated by gently massaging the tissue through a 70 μ m cell strainer (Falcon) with a 1 ml syringe plunger while bathing in several ml of ice-cold *Botryllus* buffer (50 mM EDTA, 25 mM HEPES, 10 mM cysteine in seawater, pH 7.5). Cells were pelleted at 500 \times G for 5 min at 4°C and washed in *Botryllus* buffer with 1%–2% horse serum.

Bodipy-aminoacetylaldehyde (BAAA) was kindly provided by Susan Ludeman at Duke University. Just prior to use, a 0.1 μ mol aliquot of BAAA was dissolved in 20 μ l DMSO and activated for 1.5 hr in the dark with an equal volume of 2 N HCl. Cells were stained in 1–2 μ M BAAA in *Botryllus* buffer for 20–30 min. To prevent efflux of the BAAA, Verapamil (Sigma) was included in staining and washing solutions at 2.5 mM. DEAB (Aldrich), an inhibitor of ALDH, was present in the staining media of negative controls at 100 μ M. Cells were washed twice and resuspended in *Botryllus* buffer containing 1 μ g/ml propidium iodide (Molecular Probes) and maintained on ice until FACS analysis.

Cell Sorting and Analysis

Flow cytometry was carried out on a dual laser FACStar Plus (Becton Dickinson, Mountain View, CA) equipped with a UV laser. Dead cells were excluded by gating for low propidium iodide signal. Small cellular debris that lacked nuclear material was eliminated with the use of a side scatter trigger. Analysis was performed using FlowJo software (Tree Star, Inc., San Carlos, CA). Cells for transplant were sorted into eppendorf tubes containing *Botryllus* buffer with 1% horse serum and maintained on ice. In the case of single-cell transplants, donor cells were sorted at 1:50 or 1:100 with recipient carrier cells gated for debris and propidium iodide exclusion. Within 12 hr, sorted cells were pelleted, assessed in 0.05% Trypan Blue to verify quantity and viability, and resuspended at the desired concentration in *Botryllus* buffer.

Microinjection

Glass needles were produced on a micropipet puller (Sutter Instruments) and broken at a bevel with a 50–60 μ m diameter tip. Needles were back-loaded with mineral oil and forwardloaded with a volume equivalent to the desired number of cells, corresponding to 0.2–1 μ l of fluid. Cell suspensions were mixed with 0.1% phenol red or isosulfan blue and delivered through a proximal ampulla (Figure 1). Injection was assessed by dye penetration of the surrounding vasculature, buds, and zooids. Withdrawal of blood was also performed by ampulla puncture and suction with a micropipet.

Telomerase Assay

Telomerase activity was assessed by real-time quantitative telomerase reverse-transcriptase amplification protocol (TRAP), which was developed from previous sources (Hou et al., 2001; Laird and Weissman, 2004b; Morrison et al., 1996). Cells from populations of interest were sorted into wells of an optical PCR plate (Applied Biosystems) containing 25 μ l of 1 \times SYBR green buffer (Applied Biosystems), 200 μ M dNTPs, 1 mM EGTA, 1.5 mM MgCl₂, 0.5% Tween-20, and 200 ng TS primer (5'-AATCCGTCGAGCAGATT-3'). Quantities of 10, 50, 100, 250, and 500 cells were assayed in triplicate. The plate was spun briefly and incubated for 1 hr at 37°C to lyse cells before adding 25 μ l reaction mix containing SYBR green buffer, 200 μ M dNTPs, 1 mM EGTA, 1.5 mM MgCl₂, 0.5% Tween-20, 200 ng ACX primer (5'-GCGCGG(CTTACC)₃CTAACCC-3'), and 1 U Amplitaq Gold (Applied Biosystems) and thermocycling an ABI Prism 7000 Sequence Detector System as described (Laird and Weissman, 2004b).

Genotyping

Nucleic acid was extracted from tissues in lysis buffer (5 M Guanidium Isothiocyanate, 50 mM Tris pH 7.2, 10 mM EDTA, and 1% Triton-X 100) with

5 μ l glassmilk (Bio 101, La Jolla, CA) in a protocol modified from Hoss and Paabo (1993). The glassmilk was washed with 5 M Guanidium Isothiocyanate, 50 mM Tris pH 6.4, 10 mM EDTA, then with 70% ethanol and acetone and DNA eluted at 65°C with water. DNAs from each donor-host pair were digested with EcoRI and MseI, ligated to adaptors, PCR-amplified as detailed (De Tomaso et al., 1998), and screened for polymorphic microsatellite loci (Stoner et al., 1997) or single-stranded conformational polymorphisms (SSCP) in the FuHC locus using the primer sets in Table S1. SSCP amplification was carried out in 12 μ l reactions containing 1 \times Titanium Taq buffer (Clontech), 200 μ M dNTPs, 0.4 μ M primers, 3 μ Ci ³²P- α -dATP or ³²P- α -dCTP (New England Nuclear), and 1 U Titanium Taq polymerase (Clontech) and resolved through nondenaturing 5% acrylamide for 4–8 hr at 6–15 W before exposure to X-OMAT film (Eastman Kodak).

Supplemental Data

Supplemental Data include one figure and two tables and can be found with this article online at <http://www.cell.com/cgi/content/full/123/7/1351/DC1/>.

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