cells from C3H mice are fertile and can be genetically manipulated.

Discussion

Ideally, transgenic mice with different inbred backgrounds would be useful for quantifying SSC numbers by germ cell transplantation, which allows quantification of SSCs by the counting numbers of germ cell colonies. Germ cell transplantation at two different time points would have been useful for assessing the SSC proliferation rate, as we demonstrated in B6 mice [18]. Because such transgenic mouse lines are not available, we assessed SSCs with different backgrounds by three different approaches, including histological analysis after busulfan-induced regeneration, germ cell transplantation using backcrossed transgenic mice and GS cell culture. Although whole mount or histological analyses have been traditionally used to evaluate SSCs *in situ*, a major impediment to analysis of SSCs is the inability to distinguish them from spermatogonia that are committed to differentiation. We used germ cell transplantation and GS cell culture techniques to evaluate genetic effects in SSC self-renewal to complement the results of histological analyses.

Our initial set of experiments demonstrated a wide difference in regeneration after busulfan treatment. Using the germ cell transplantation technique, we previously quantified changes of total SSC number per testis during busulfan-induced regeneration. We found that busulfan administration depleted the number of SSCs to 4% of those in the wild-type testis within 3 days, but the numbers of SSCs in B6 mice gradually regenerated to 61% by 70 days [18]. Likewise, regeneration of spermatogenesis occurred in all of the tested strains. Spermatogenesis increased between 35 and 70 days after treatment in all cases, suggesting a gradual recovery of the SSC population after busulfan treatment. However, the degrees and the patterns of regeneration were significantly different among the strains. Spermatogenesis in DBA and AKR mice was relatively resistant to busulfan, and 60–80% of their tubules contained multiple layers of germ cells by 35 days. This differed from spermatogenesis in BALB and C3H mice, which were highly sensitive to busulfan; nonetheless, spermatogenesis recovered dramatically between 35 and 70 days. Spermatogenesis in B6 mice appears to have characteristics that fall somewhere between these two types; whereas it was apparently more sensitive to busulfan than those in DBA/AKR group, the regeneration potential was more limited than those in BALB/C3H group.

However, these results do not necessarily reflect the number and self-renewal potential of SSCs. Although aging does not affect spermatogenic recovery after busulfan treatment [32], regeneration of spermatogenesis involves additional parameters other than SSC self-renewal, including absorption and metabolism of busulfan and damage to other cell components such as Sertoli or Leydig cells. Chemotherapeutic treatments in other studies showed that testes that retain spermatogonia on the basement membrane are unable to undergo spermatogenesis [33]. SSC self-renewal is also influenced by the presence of differentiated cells. It has previously been shown that undifferentiated spermatogonial proliferation becomes greatly enhanced when the number of differentiating spermatogonia gets below a threshold level after administration of busulfan [34]. This may explain why spermatogenesis recovered much faster in mice that seemed most vulnerable to busulfan, such as BALB and C3H. Furthermore, it also depends on the immediate sensitivity of SSCs to busulfan, which influences the initial SSC number before they start regeneration. Thus, while these results suggest significant strain differences in the self-renewal activity of SSCs, busulfan-induced regeneration involved many parameters that are not relevant to SSC self-renewal, and the results from the first set of experiments did not conclusively demonstrate that SSCs with AKR or DBA backgrounds proliferate more actively.

Germ cell transplantation experiments have clearly shown the regenerative potential of SSCs. Several germ cell transplantation studies have established that a single colony is derived from a single SSC [7, 26, 27], and that the germ cell colony count is proportional to the number of transplanted SSCs [35]. Therefore, the number of SSCs can be quantified by introducing a donor cell marker and counting the number of germ cell colonies. Using EGFP-labeled donor cells, we compared SSC proliferation for two genetic backgrounds under the same *in vivo* conditions. The total number as well as the frequency of SSCs in the testis cell suspension was not significantly different between the B6 and DBA mice. The SSC frequency in the total testis cell suspension (0.01–0.06% and 0.01–0.02% in the DBA and B6 mice, respectively) was comparable to previous estimates obtained by morphological analysis (0.02–0.03%)[1, 36]. Therefore, the rapid recovery of spermatogenesis in the DBA background is probably not due to increased SSCs in this strain. Although we did find more SSCs in the DBA mice, the difference in the SSC pool size may not be quite as pronounced as found in the hematopoietic system, where the size of the total stem cell pool in DBA mice was several times larger than that in B6 mice [15]. It is unclear whether this is caused by differences in the self-renewing machinery or whether it can be attributed to limitations of the stem cell assays in the two different self-renewing systems. Regardless, it is of interest and needs to be examined from different angles in future analyses.

Serial transplantation experiments confirmed the effect of genetic factors on SSC proliferation. By comparing the numbers of germ cell colonies between the primary and secondary recipients, it is possible to measure the net increase of SSCs in the primary recipient over the course of 2 months because single germ cell colonies were originally produced by single SSCs that underwent self-renewal divisions. Based on colony counts (0.86 *vs.* 0.35), our estimates indicate that the SSCs in the DBA mice produced 2.5-fold more SSCs than those in the B6 mice. A genetic influence on spermatogenic activity has been suggested in several studies, including pioneering observations on experimental chimeras in the 1960s [37–39]. In male C3H and B6 chimeric mice, the proportion of the B6 progeny decreased markedly, while the total litter size remained constant. This selective shift with age of the males was thought to occur at the spermatogonia stage, and it was suggested that spermatogonia of the two strains might proliferate at different rates [38]. However, it has remained unclear whether the shift is caused by differences in the self-renewal activity of SSCs or is due to proliferation of differentiated progenitor cells. Our results suggest that genetic factors play an important role in SSC self-renewal *in vivo* and may explain the phenomenon observed in those chimeric mice.

The current results indicate that SSC self-renewal is affected by the genetic background; however, the derivation of GS cells may involve additional factors. We reported that GS cells could be readily established from DBA, B6D2F1 or ICR backgrounds [8]. Although similar cells were reported from other genetic backgrounds including B6 and 129, they grew poorly in culture, and no genetic manipulation of these cells has been reported [13]. In the present study, we were able to establish GS cells from C3H mice, which were sensitive to busulfan-induced damage but responded in a manner distinct from DBA or B6 mice. These GS cells possessed SSC potential, and successful production of transgenic offspring from GS cells of C3H mice indicated that GS cell-based techniques can be applied to a wide range of strains. However, GS cells from the B6 background did not proliferate actively under the same culture conditions, and we have not been able to produce transgenic offspring using plasmid vectors in these mice (our unpublished observation). Thus, there is clearly a need to improve current culture conditions. Although the addition of GFRA1 has been reported to enhance SSC self-renewal [13], it did not have a strong impact in our culture system. The search for additional self-renewal factors must be continued in order to improve the efficacy of the GS cell culture technique.

There are multiple variables that make it difficult to study the genetic differences in self-renewal of SSCs. These include strain differences in 1) sensitivity to busulfan, 2) absolute numbers of endogenous SSCs at any one time, 3) the rate of self-renewal of endogenous SSCs and 4) interactions between SSCs and niche cells or any other somatic cell type that might influence self-renewal of SSCs. The effects of these factors could not be neglected totally in this study. Because SSCs are defined only by their ability to self-renew, they must be assessed by germ cell transplantation, which is the only method available to detect SSCs in a functional manner. We attempted to study the genetic differences of SSCs using this technique. Albeit with several caveats, this study provided a basis for study of the genetic differences of SSCs by functional methods. It also raises several important questions for understanding the kinetics of spermatogenesis. How genetic factors influence SSC behavior *in vivo*, including the regulation of pool size, the life-span and the stress response, remains unknown. For example, studies in the hematopoietic system have revealed fundamental differences between the mechanisms that control the frequency of HSCs and those that regulate mature blood cell numbers [15]. Understanding these regulatory factors is important for elucidating the molecular basis of SSC self-renewal. It is also important to conduct genetic mapping to find genes that influence SSC self-renewal. At present, we only know of a few molecules that promote SSC self-renewal. The analysis of genetic factors may help to identify additional components that modulate SSC self-renewal.

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