Glycolysis-Optimized Conditions Enhance Maintenance of Regenerative Integrity in Mouse Spermatogonial Stem Cells during Long-Term Culture

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SUMMARY

The application of spermatogonial stem cell (SSC) transplantation for regenerating male fertility requires amplification of SSC number in vitro during which the integrity to re-establish spermatogenesis must be preserved. Conventional conditions supporting proliferation of SSCs from mouse pups have been the basis for developing methodology with adult human cells but are unrefined. We found that the integrity to regenerate spermatogenesis after transplantation declines with advancing time in primary cultures of pup SSCs and that the efficacy of deriving cultures from adult SSCs is limited with conventional conditions. To address these deficiencies, we optimized the culture environment to favor glycolysis as the primary bioenergetics process. In these conditions, regenerative integrity of pup and adult SSCs was significantly improved and the efficiency of establishing primary cultures was 100%. Collectively, these findings suggest that SSCs are primed for conditions favoring glycolytic activity, and matching culture environments to their bioenergetics is critical for maintaining functional integrity.

INTRODUCTION

In mammalian testes, spermatogonial stem cells (SSCs) are a rare subset of the undifferentiated spermatogonial population that is tasked with providing continuity to the spermatogenic lineage (Oatley and Brinster, 2008, 2012). In addition, SSCs possess regenerative capacity, and following isolation from a donor testis can be transplanted into germ cell-depleted recipient testes to re-establish spermatogenesis. The transplantation paradigm has been demonstrated in rodent and non-human primate models (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994; Hermann et al., 2012). Although the methodology has not yet been translated to humans, autologous transplantation of SSCs isolated from a testicular biopsy prior to chemotherapy is a potential treatment for re-establishing fertility in both adult and adolescent cancer patients (Kubota and Brinster, 2006). However, due to their rarity, achieving fertility restoration by transplanting the few SSCs collected from a biopsy is unlikely. Therefore, in vitro expansion of SSC numbers is critical for clinical application of transplantation.

Devising culture conditions for human SSCs is limited by lack of an assay to directly assess survival and growth of the cells. Indeed, previous studies have relied on the use of molecular markers and xenotransplantation of cultured human testis cells into the seminiferous tubules of immune deficient mice to assess whether SSCs were present (Nagano et al., 2002; Ramathal et al., 2014, 2015; Sadri-Ardekani et al., 2009, 2011). These approaches do not provide definitive measures of SSCs because molecular markers specific for regenerative spermatogonia in humans are undefined and even though presumptive germ cell colonies composed of short chains of cells form in xenotransplantations, the spermatogenic lineage is not fully regenerated, thereby limiting utility of the assay for assessing SSC integrity directly.

In contrast to human germ cell cultures, primary cultures of mouse undifferentiated spermatogonia can be established and maintained for extended periods of time in relatively minimal conditions. All culture methodology reported to date is based on standard conditions that include: (1) chemically defined or complex StemPro-based serum-free medium; (2) supplementation of base medium with a growth factor cocktail of glial cell line-derived neurotrophic factor (GDNF), fibroblast growth factor 2 (FGF2), and a lipid or free fatty acid mixture; (3) co-culture with mitotically inactivated somatic feeder cells (MEF [mouse embryonic fibroblast], STO [SIM mouse embryo-derived thiguanine and ouabain resistant], or testicular stromal cells); and (4) maintenance in an atmosphere of 5% CO2 and 21% O2 tension. A multitude of studies have demonstrated maintenance of heterogeneous undifferentiated spermatogonial populations in these conditions that are composed of a small number of SSCs and abundant non-stem cell progenitor spermatogonia (Kanatsu-Shinohara et al., 2003, 2011; Kubota et al., 2004b; Oatley et al., 2009; Schmidt et al., 2011; Seandel et al., 2007; Takashima et al., 2015). Importantly, a robust transplantation assay is in place to directly assess the maintenance of SSC regenerative integrity in the primary cultures (Helsel and Oatley, 2017). For these reasons, cultures of mouse cells are an attractive platform for optimizing conditions that may be applicable to a wide range of other mammalian species including humans. Although in place for over a decade, understanding of the impacts of conventional conditions on
regenerative integrity during long-term culture is still limited. While previous studies have assessed the ramifications of long-term culture on maintenance of genetic and epigenetic parameters, the outcomes are difficult to interpret, especially in the context of regenerative medicine. Studies by Kanatsu-Shinohara et al. (2005) explored expansion of SSC numbers and epigenetic imprinting in primary mouse cultures over a period of 2 years. Outcomes revealed no alterations in the imprinting patterns of three paternally methylated regions and two maternally methylated regions (Kanatsu-Shinohara et al., 2005). However, considering that the cultures examined are a heterogeneous mix of rare SSCs and much more abundant progenitors, interpreting these findings in regard to SSCs specifically is challenging. In addition, a nuance of those studies is that cultures were established from postnatal day 0 (P0) pups, an age point in development where the germ cell population comprises prospermatogonial precursors that have yet to transition to SSCs. Considering that the prospermatogonia-to-SSC transition happens prior to birth in humans (Ginsberg et al., 2010), the relevance of these findings to culture of SSCs isolated from prepubertal boys or men is not clear. Indeed, studies by Schmidt et al. (2011) reported a significant decline in the number of SSCs in primary cultures that were established from P6–P8 pups over a period of 18 months without a concomitant alteration in DNA methylation at imprinted genes. Thus, although previous studies have provided some insight into the persistence of SSCs in short-term cultures, the question of whether SSC regenerative integrity is preserved has not been fully addressed.

A limited number of studies have evaluated primary cultures of undifferentiated spermatogonia established from adult mice (Schmidt et al., 2011; Seandel et al., 2007), but the relative SSC content and regenerative integrity over an extended period of in vitro proliferation have not been reported. Indeed, a majority of studies regarding culture of rodent SSCs utilized testis cells isolated from prepubertal or neonatal mice. However, studies by Seandel et al. (2007) reported persistence of SSCs over a 2-year period in cultures established from adult testis cells, although quantification of SSC content at various points throughout the culture period were not provided. Moreover, maintenance of regenerative integrity to re-establish the entire spermatogonial lineage was not assessed. Because parameters associated with cultures from adult mice are reflective of establishing cultures from men, assessing the efficiency by which they can be established and maintenance of SSC regenerative integrity is imperative.

In the present study, we found a negative association between time of maintenance and regenerative integrity of SSCs in primary cultures of undifferentiated spermatogonia established from cells isolated from testes of pup and adult mice that were maintained in conventional conditions. To address the deficiency, we sought to refine culture conditions based on our understanding of the biochemistry of SSCs. Our previous studies revealed that expression of key enzymes that regulate glycolysis are elevated in SSCs of primary undifferentiated spermatogonia cultures (Chan et al., 2014). Thus, we examined the impacts of modifying culture conditions to favor utilization of glycolysis as the primary bioenergetics process. When maintained in glycolysis-optimized (GO) conditions, the capacity of SSCs to regenerate complete colonies of spermatogenesis was significantly improved compared with conventional conditions for both pup and adult primary cultures. These findings suggest that a distinguishing characteristic of SSCs is priming to thrive in conditions favoring glycolytic activity, and matching culture conditions to the bioenergetics of the cells is important for maintaining functional integrity.

RESULTS

Changes in the SSC Content of Primary Cultures of Undifferentiated Spermatogonia during Long-Term Maintenance in Conventional Conditions

To determine the impact of long-term in vitro maintenance on the integrity of SSCs, we first utilized primary cultures of undifferentiated spermatogonia established from P6–P8 Rosa26-LacZ transgenic pups. The cells were maintained in conventional conditions that included a chemically defined serum-free medium supplemented with a free fatty acid cocktail, GDNF, and FGF2, and co-culture with STO feeder cells in an atmosphere of 5% CO2 and 21% O2 (Kubota et al., 2004b). For each culture analyzed, the spermatogonial population was collected at 2-month intervals and a portion (~1 × 10⁵ cells) was transplanted as a single-cell suspension into the testes of germ cell-depleted recipient mice (1 × 10⁴ cells transplanted per testis) to assess SSC content, while the remaining cells were replated for continued culture (Figure 1A). Based on colonies of LacZ-stained donor cells in recipient seminiferous tubules, the mean (±SEM) relative SSC content of cultures at 2, 4, and 6 months (n = 4 or 5 cultures analyzed at each time point) was calculated to be 202.0 ± 72.6, 192.6 ± 51.0, and 15.4 ± 8.9, respectively (Figures 1B and 1C), demonstrating a decrease of ~13-fold over the culture period. However, the total number of spermatogonia increased exponentially throughout the 6-month period (Figure 1D). Thus, a decrease in overall growth of the culture was not the reason for the declining SSC content. Importantly, these findings corroborate those of Schmidt et al. (2011), which also demonstrated significant decline of SSC
Assessment of SSC Regenerative Integrity in Primary Cultures of Undifferentiated Spermatogonia during Long-Term In Vitro Maintenance

The regenerative integrity of cultured SSCs can be defined as a capacity to produce robust colonies of continual spermatogenesis in recipient seminiferous tubules following transplantation. From a human clinical standpoint, sustaining this capacity following culture is critical if restoration of fertility is the desired outcome of transplantation. From the transplantation analyses for assessing changes in SSC content of long-term cultures presented above, we observed donor-derived colonies with variable morphologies at all time points. Some colonies appeared to contain the entire spermatogenic lineage, as indicated by dense blue staining, while others were sparsely populated with germ cells, as indicated by patchy staining in which individual cells were distinguishable (Figure 2A). To explore these observations further, we transplanted cells from 2-month cultures into testes of W/Wv recipients that lack an endogenous germline, thereby allowing us to unequivocally assess the degree of regenerated spermatogenesis arising from cultured and transplanted donor SSCs specifically. Approximately 12 weeks after transplant, which constitutes greater than two rounds of spermatogenesis, recipient testes were recovered and processed for histological analysis of serial cross-sections through the entire tissue to define complete and incomplete colonies. As expected, colonies of complete spermatogenesis were evident in some seminiferous tubule cross-sections; however, we also observed many tubules with incomplete spermatogenesis (Figure 2B). Further assessment of the incomplete colonies revealed that 44.6% ± 3.1% (mean ± SEM) contained spermatogonia only and 55.4% ± 3.1% contained at least some meiotic spermatocytes (Figure 2C). Seminiferous tubules containing round spermatids or spermatozoa were not evident in any of the incomplete colonies examined (n = 21 cross-sections). Immunofluorescent staining for the marker TRA98 confirmed that all incomplete colonies contained germ cells, and staining for the marker LIN28 demonstrated that all contained undifferentiated spermatogonia (Figure 2D). Because each colony from transplantation is clonally derived from an individual SSC (Kanatsu-Shinohara et al., 2006; Nagano et al., 1999), these findings demonstrated that all SSCs in primary cultures of undifferentiated spermatogonia do not possess equivalent capacity to regenerate the entire spermatogenic lineage. Also, these findings suggest a multifaceted deficiency in long-term cultured SSCs that manifests as an arrest at various steps in the spermatogenic continuum, including spermatogonial differentiation and meiotic progression.

Next, we sought to determine whether the regenerative integrity of the SSC population changes during long-term maintenance in conventional culture conditions. To address this, we examined the intact LacZ-stained recipient testes that had been transplanted with cultured cells at 2, 4, and 6 months and scored the colonies within whole seminiferous tubules as complete or incomplete based on dense blue staining or being sparsely populated with blue-stained content during long-term culture of >5 months in conventional conditions.

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cells, respectively. The mean percentage of complete colonies was 79%, 26%, and 5% at 2, 4, and 6 months of culture, respectively (Figure 2E). Thus, between 2 and 6 months in culture a loss of 16-fold in the number of SSCs with the capacity to regenerate the entire spermatogenic lineage occurred. These findings demonstrate a major decline in regenerative integrity of the SSC population during long-term maintenance in conventional culture conditions, which imposes a major limitation on translating the current methodologies devised for mice to culture of human SSCs.

Impact of Culture Conditions Optimized for Utilization of Glycolysis as the Primary Bioenergetics Process on SSC Regenerative Integrity during Long-Term In Vitro Maintenance

To begin addressing the deficiency of conventional conditions for maintaining the regenerative integrity of long-term cultured SSCs, we mined the transcriptome profile of the ID4-EGFP+/SSC pool from primary mouse cultures that was generated in our previous studies (Chan et al., 2014). This assessment uncovered upregulated expression of genes coding for the enzymes enolase 1 and 2 (Eno1/2) in the ID4-EGFP+/SSC population compared with the ID4-EGFP+/progenitor population. We confirmed upregulated expression of ENO2 in the ID4-EGFP+/SSC pool isolated by fluorescence-activated cell sorting from primary cultures using immunoblot analysis (Figure S1). Because ENO1/2 play key roles in the penultimate step of glycolysis, we postulated that the SSC pool in primary cultures is primed to thrive in conditions that favor glycolysis as the primary bioenergetics process. To assess this possibility, we modified culture conditions to provide a GO environment by removing all sources of lipids and free fatty acids from culture medium and lowering the O2 tension of the atmosphere from the standard atmospheric 21% to 10%. Based on lactate levels, we found that cells maintained for as little as one week in GO conditions had elevation of 32% (n = 3 different cultures) in glycolytic activity compared with cells maintained in conventional conditions (Figure S2). Next, cultures were established in GO conditions from P6–P8 Rosa26-LacZ transgenic pups (n = 4) and maintained for a 6-month period. At 2-month intervals, a portion of the cell population was collected for transplantation into germ cell-depleted recipients to measure SSC content and regenerative integrity, and the remaining cells were replated for continued culture. Outcomes revealed the mean (±SEM) relative SSC content of the cultures to be 150.0 ± 47.7, 170.5 ± 47.3, and 29.5 ± 11.2 per 105 cells, respectively (Figures 3A and 3B). Although the SSC content was numerically lower compared with conventional conditions, the difference was not statistically significant (p = 0.7). Also, even though
a decrease in overall SSC content still occurred in GO conditions over the 6-month period (~5-fold), it was appreciably less compared with the major decline (~13-fold) in conventional conditions (Figure 1C).

Next, we aimed to determine whether GO conditions affect the regenerative integrity of SSCs during long-term culture. To address this, we scored spermatogenic colonies that formed in recipient testes 2 months after transplantation with cells cultured in GO conditions for 2, 4, or 6 months as being complete or incomplete using the criteria defined above. For 2-month cultured cells (n = 4), we found that the mean percentage of regenerated colonies that could be scored as complete was ~76%, which was similar to that for cells cultured in conventional conditions for 2 months (Figures 3C and 2E). However, in contrast to the major decline of cells cultured in conventional conditions for 4 months (~26% complete colonies) or 6 months (~5% complete colonies), the percentage of complete colonies produced from cells cultured in GO conditions for 4 or 6 months was 44% and 40%, respectively (Figures 3C and 2E). Although a decline of ~2-fold occurred from 2 to 6 months of maintenance, the number of SSCs in primary cultures with the capacity to fully regenerate the spermatogenic lineage in GO conditions was improved by ~70% and ~800% at 4 and 6 months of culture, respectively, compared with conventional conditions. Taken together, these findings suggest that matching the culture environment to the primary bioenergetics of SSCs is critical for preserving regenerative integrity.

**Glycolysis-Optimized Conditions Enhance Long-Term Culture of SSCs from Adult Mice**

Although a few previous studies have reported long-term maintenance of primary cultures of undifferentiated spermatogonia from adult mice (Ogawa et al., 2004; Schmidt et al., 2011; Seandel et al., 2007), the efficiency of establishing and preservation of SSC regenerative integrity has not been evaluated. Here, we compared the efficiency of establishing long-term primary cultures of undifferentiated spermatogonia from adult mice in conventional or GO conditions. For these experiments, adult *Rosa26-LacZ* donor mice at 3–4 months of age were utilized as donors. For each, the isolated spermatogonial population was divided equally in number for maintenance in conventional or GO conditions. A culture was considered successfully established when the overall cell number expanded to the point where it could be split 1:2 with continued growth. Overall we were able to establish two cultures out of ten attempts for conventional conditions, but six out of six attempts were successful with the GO conditions. In addition, the average time to achieve exponential expansion for the two successful conventional cultures was ~6 months, whereas GO cultures achieved this by ~2 months of maintenance. Similarly to the pup cultures, lactate levels were measured to be increased (~56%) in adult cells maintained in GO compared with conventional conditions indicating enhanced glycolytic activity (Figure S2).

Next, we aimed to assess the maintenance of SSC content and regenerative integrity in long-term adult cultures. For GO conditions, the cell populations were collected at 2-month intervals throughout a 12-month period and a portion (~1 x 10^5 cells) was transplanted into the testes of recipient mice to assess SSC content while the remaining cells were replated for continued culture. Because the time to reach exponential expansion was delayed for cultures in conventional conditions, transplantation analyses were conducted at 6, 8, 10, and 12 months of culture. Outcomes for the conventional cultures revealed the relative SSC
content to be 203 (n = 1), 183.2 ± 104.8 (mean ± SEM, n = 2), 187.5 ± 107.5 (mean ± SEM, n = 2), and 6 (n = 1) per 10^5 cells transplanted at 6, 8, 10, and 12 months, respectively (Figures 4A and 4B). For GO cultures (n = 4), the mean (±SEM) relative SSC content was 286.0 ± 60.8, 278.2 ± 87.1, 163.8 ± 63.7, 136.6 ± 27.9, 130.0 ± 49.9, and 108.5 ± 26.5 SSCs per 10^5 cells transplanted at 2, 4, 6, 8, 10, and 12 months of culture, respectively (Figures 4A and 4B). Although the relative SSC content of the adult cultures was greater than that in pup cultures for both GO and conventional conditions, a decline of ~2-fold between 2 and 6 months and ~3-fold over the full 12-month period occurred for GO conditions, similarly to pup cultures. Lastly, we assessed the regenerative integrity of SSCs in the adult cultures during long-term maintenance by scoring donor-derived colonies generated in recipient testes as either complete or incomplete. For conventional cultures ~50% of the colonies were complete at 6 months (n = 1), dropping to ~11% (n = 2) at 8 months, reducing even further to 2% (n = 2) at 10 months, and by 12 months 0% of colonies contained complete spermatogenesis (Figure 4C). In contrast, ~89%, 69%, 50%, 40%, 8%, and 2% (n = 4) of colonies were complete at 2, 4, 6, 8, 10, and 12 months of culture, respectively (Figure 4C). Thus, GO conditions support an SSC pool in primary cultures of undifferentiated spermatogonia derived from adult testes for at least 6 months in which regenerative integrity is preserved in ~50% of the population.

**DISCUSSION**

Expansion of SSC numbers in vitro while maintaining the integrity to regenerate complete colonies of spermatogenesis is paramount for application of transplantation methodology as a means to preserve and re-establish the fertility of male cancer patients. Because the proliferation rate of SSCs is relatively slow, ~6 days (Kubota et al., 2004b), a period of long-term maintenance in culture is needed to provide a sufficient number of cells for transplantation if the intent is to yield enough sperm for a pregnancy to be achieved. In addition, long-term culture of SSCs provides a valuable platform to study the mechanisms controlling their fate decisions. Several studies have reported long-term maintenance of putative human SSCs (Sadri-Ardekani et al., 2009, 2011). Although the outcomes were promising,
unequivocal determination of SSC presence in human testicular cell cultures is challenging because assessment is restricted to expression of molecular markers whose SSC specificity is undefined and the appearance of human cells in testes of immunodeficient recipient mice after xenotransplantation. Although some of the markers used are expressed by somatic cells, others are expressed by spermatogonia but are not SSC specific. Also, xenotransplantation does not result in regeneration of the spermatogenic lineage; thus, whether bona fide SSCs were present in the human cultures has not been truly demonstrated.

In previous attempts to culture human SSCs, conditions were based on methodology described previously for long-term culture of primary mouse undifferentiated spermatogonial populations in which the presence of stem cells was demonstrated by transplantation analyses that yielded complete regeneration of spermatogenesis. In all mouse conditions described to date, the primary population of spermatogonia consists of an SSC pool that is small in number and a more abundant non-SSC population that is equivalent to transit amplifying progenitors in vivo. However, conditions for maintenance of the SSC pool have not been optimized. Importantly, availability of the robust transplantation assay to assess maintenance of SSC numbers and their regenerative integrity is a major benefit for refining conditions, which can yield invaluable information for developing human SSC culture methodology.

Two major conventional methodologies for long-term culture of mouse undifferentiated spermatogonial populations that contain bona fide SSCs were described over 10 years ago. Studies of Kanatsu-Shinohara et al. (2003) developed methodology that includes a complex medium formulation based on the commercial supplement StemPro and co-culture with MEF feeder cells, which supports long-term maintenance and growth of a primary undifferentiated germ cell population derived from testes of neonatal (P0) mice. Although an exciting breakthrough, these cultures were derived from prospermatogonia that are present at P0, which are precursors to undifferentiated spermatogonia. Also, the conditions were developed for mice with a DBA/2 genetic background in which adult stem cells are known to possess enhanced capacity for proliferation and survival compared with other strains of mice (Müller-Sieburg and Riblet, 1996). The relevance of these conditions to actual undifferentiated spermatogonia including SSCs in either prepubertal pups or adults and other strains of mice has remained relatively undefined. Subsequently, Kubota et al. (2004b) developed a method that includes a defined medium formulation and co-culture with STO feeder cells that supports long-term maintenance and growth of a primary undifferentiated spermatogonial population derived from P6–P8 mice of several genetic backgrounds including DBA/2 and C57BL/6. In contrast to claims by Kanatsu-Shinohara et al. (2016) that derivation of cultures is notoriously difficult with mice of a C57BL/6 background, a multitude of studies have utilized the method of Kubota et al. (2004b) to derive long-term cultures of cells from P6–P8 mice with this genetic background and utilize them successfully for experimentation of the SSC pool (Chan et al., 2014; Kaucher et al., 2012; Lovelace et al., 2016; Oatley et al., 2006, 2007, 2009, 2010, 2011; Wu et al., 2010; Yang et al., 2013a, 2013b, 2013c). It is important to note that for both of the conventional methods, only a minor portion of the germ cell population possesses the capacity to regenerate spermatogenesis following transplantation into recipient testes. Hence, the cultures cannot be considered pure SSCs or germline stem (GS) cells. In actuality, the cultures are a primary population of undifferentiated spermatogonia if derived from prepubertal pups and prospermatogonia if derived from P0 neonates.

A nuance of the conventional conditions has been that while overall amplification of cell number occurs in the cultures, the percentage of the population possessing regenerative capacity (i.e., SSCs) declines over a several-month period (Kanatsu-Shinohara et al., 2003, 2005, 2011; Kubota et al., 2004b; Schmidt et al., 2011), a deficiency that is amplified when the germ cells are maintained in a feeder-free format (Kanatsu-Shinohara et al., 2011). There are several possible causes of the declining SSC content, including dilution of the pool by greater proliferation of the non-stem cell progenitor spermatogonia or impaired integrity of SSCs to self-renew. At present, experimental evidence supporting a singular or multifactor deficiency has not been generated, and further experimentation will be required to clarify the cause of reduced maintenance of the SSC pool. Also, the integrity of the colony-producing population for regenerating complete spermatogenesis at various points during the culture period has not been defined. In an effort to enhance growth of the cultures overall and improve maintenance of the SSC pool, Kanatsu-Shinohara et al. (2011) refined the StemPro-based culture medium to be serum free and lipid enriched. In those conditions, the growth of cultures overall was improved and a pool of cells with regenerative capacity persisted for an extended period of time. In follow-up studies, the same group aimed to improve the serum-free and lipid-rich condition by changing the commercially available base medium (Kanatsu-Shinohara et al., 2014). Outcomes yielded a condition whereby the percentage of the culture population that is capable of regenerating spermatogenesis was maintained at a relatively steady level over a 5-month period. However, for both of these refined conditions, assessment of the percentage of the population with prime stem cell capacity to produce colonies of complete spermatogenesis after transplant at various points during the culture period was not conducted. Also, similar to the...
unrefined conditions, the cultures were derived from prospermatogonia of P0 neonates with a DBA/2 genetic background; thus, relevance to the true undifferentiated spermatogonial population and other strains of mice has remained undefined.

For many somatic cell lineages, the bioenergetics of stem cells is unique compared with progenitors and differentiated cells, with most relying on glycolysis (Hsu et al., 2013; Ito and Suda, 2014; Simsek et al., 2010). If this is the case for SSCs, modifying conditions to favor glycolysis could be of benefit for maintaining regenerative integrity of the pool during long-term culture. Indeed, our previous studies that examined the transcriptome of the SSC pool in primary cultures of undifferentiated spermatogonia derived from P6–P8 mice with a C57BL/6 genetic background revealed differential expression of key enzymes (Eno1/2) that are involved in glycolysis (Chan et al., 2014). Expanding on this observation, a recent study found that inhibition of glycolysis in prospermatogonia-derived cultures derived from DBA/2 mice reduced the number of cells with regenerative capacity, whereas chemical activation of glycolysis enhanced stem cell maintenance (Kanatsu-Shinohara et al., 2016). Paradoxically, because an abundance of free fatty acids will promote utilization of β-oxidation, the findings that modulating glycolytic activity improves SSC self-renewal contradicts the previous studies suggesting that a lipid-rich medium enhances SSC maintenance during long-term culture (Kanatsu-Shinohara et al., 2011, 2014). Unfortunately, the SSC content was not reported at various points during an extended culture period in the study by Kanatsu-Shinohara et al. (2016), so the impact of modulating glycolytic activity on long-term maintenance of the SSC pool is not clear. Interestingly, by adding a chemical activator of glycolysis to culture medium, Kanatsu-Shinohara et al. (2016) were able to derive primary cultures from mice with a C57BL/6 genetic background, a feat they had not been able to achieve using the StemPro and lipid-rich condition that works efficiently for cells from DBA/2 mice. Although the percentage of the cultured populations that possessed regenerative capacity was reported to be similar between C57BL/6 and DBA/2 cultures when maintained with supplementation of the glycolysis activator, the length of time that the cells had been maintained in vitro prior to transplant was not reported. In addition, the percentage of colonies that formed in recipient testes after transplantation that were complete spermatogenesis was not assessed. Thus, the effects of modulating the bioenergetics in primary cultures of undifferentiated spermatogonia on long-term maintenance of SSC regenerative integrity have remained undefined.

While all of the original and refined conventional conditions appear to support primary germ cell populations derived from testes of neonatal or prepubertal pups in which at least some SSCs persist for various periods of time, the impacts of the conditions on derivation and long-term maintenance of SSC containing cultures from adults have not been thoroughly explored. For the methodology reported by Kubota et al. (2004b), a single study succeeded in culturing adult SSCs (Schmidt et al., 2011), but the efficiency by which the cultures could be established was not assessed and the relative SSC content was examined at only a single time point. To our knowledge, only two other groups have demonstrated success with culture of adult mouse SSCs. Previous studies by Ogawa et al. (2004) reported a success rate of 50% for adult DBA/2 mice in deriving cultures of cells with the morphological appearance of spermatogenesis when utilizing the complex StemPro-based medium reported by Kanatsu-Shinohara et al. (2003). Unfortunately, as transplantation analyses were not conducted with the cultures, the actual stem cell content of the cultures was undefined. Similarly, studies by Seandel et al. (2007) reported a culture method using testicular stromal cells as feeders and a variation of the complex StemPro-based medium originally reported by Kanatsu-Shinohara et al. (2003) that supports long-term expansion of a primary population of spermatogonia isolated from adult testes. Although data were not provided, the efficiency of establishing cultures from adult mice with various genetic backgrounds was reported to be high. Importantly, colonies of spermatogenesis were generated in recipient testes following transplantation of the cells, thus demonstrating that a portion of the population possessed SSC capacity. However, the number of colonies generated from the adult cultures was not reported, nor were transplantation analyses conducted at multiple points throughout the culture period. Therefore, assessing effectiveness of the culture method for long-term maintenance of the SSC pool is not possible at this time. Consequently, a void exists for robust and reproducible methodology to establish and maintain long-term primary cultures of adult SSCs.

For all of the culture conditions that support long-term maintenance of a primary germ cell population reported to date, the regenerative integrity of SSCs to re-establish complete colonies of spermatogenesis has not been thoroughly evaluated. It is essential to address this gap in knowledge if the intent of cultures is to re-establish fertility following transplantation. In the current study, we discovered that the percentage of SSCs that could regenerate complete colonies of spermatogenesis declines rapidly in cultures established from prepubertal pup spermatogonia and maintained in conventional conditions (Figure 1). A potential major problem is the accumulation of DNA damage in cultured SSCs, which could lead to induction of apoptosis or maturation arrest in descendant germ cells that arise in colonies following transplantation, thereby
eliminating the development of complete spermatogenesis. In this regard, generation of low levels of reactive oxygen species (ROS) during long-term culture would be critical. Indeed, outcomes of previous studies suggest that SSC maintenance in vitro is influenced by ROS (Morimoto et al., 2013, 2015). Although those studies were conducted with primary cultures derived from spermatogonia, results of manipulating ROS levels revealed that a moderate concentration promotes self-renewal. Thus, modulating ROS levels in cultures of primary spermatogonia is likely important for maintenance of SSC regenerative integrity, and a possible means of achieving this is to maintain cells in conditions that reduce the utilization of β-oxidation as the primary bioenergetics process. Our previous finding that expression of Enol1/2 is upregulated in cells that make up the SSC pool in primary cultures of pup undifferentiated spermatogonia indicated a priming to thrive in conditions that favor glycolysis as the primary bioenergetics process. All of the culture conditions reported in previous studies have maintained cells in lipid-rich or free fatty acid-supplemented media and an atmospheric O2 tension of 21%, which likely promotes the utilization of β-oxidation.

In the current study, we devised simple refinements to the conventional culture condition of Kubota et al. (2004b) that promotes utilization of glycolysis, which we have termed GO (glycolysis-optimized) conditions. To achieve this, we removed all supplementation of lipids and free fatty acids from the medium and reduced the O2 tension to 10%. In these conditions, we found that the integrity of long-term cultured SSCs from both pup and adult mice to regenerate complete colonies of spermatogenesis was significantly improved. Considering that reduced O2 tension is known to enhance the maintenance of somatic stem cell populations in vitro (Csete, 2005; Ishikawa and Ito, 1988; Maeda et al., 1986), it is possible that the reduced O2 level of the GO condition alone was the major influential factor in improving SSC regenerative capacity. However, omission of lipids in addition to the reduced O2 tension did not negatively affect maintenance of the SSC pool during long-term culture for either pup or adult cells. Thus, we recommend that both these modifications be employed in future studies utilizing primary cultures of mouse SSCs and anticipate that they will serve as the basis for developing culture conditions for SSCs from higher-order mammals. Indeed, we discovered in a previous study that long-term culture of cattle undifferentiated spermatogonia is enhanced in an atmosphere of reduced O2 tension (Oatley et al., 2016). In addition, the findings of the current study highlight the importance of matching culture conditions to the bioenergetics of SSCs during long-term in vitro maintenance and provide the impetus for future studies to further refine the conditions to promote utilization of glycolysis.

**EXPERIMENTAL PROCEDURES**

**Animals**

All animal procedures were approved by the Washington State University Institutional Animal Care and Use Committee in accordance with Guide for the Care and Use of Laboratory Animals, eighth edition. Donor mice for primary cultures of undifferentiated spermatogonia were male Rosa26-LacZ (Jax Mice, strain 002073) hybrids at P6–P8 or adult (3–4 months) ages. Recipients for transplantation analyses were either adult male W/Wv mutants (Jax Mice, strain 000499) or F1 hybrids of a C57BL/6j (Jax Mice, strain 000664) × 129S1/svImJ (Jax Mice, strain 002448) cross that were treated with busulfan to eliminate the endogenous germline, as described previously (Helsel and Oatley, 2017; Oatley and Brinster, 2006).

**Cell Culture**

Primary cultures of undifferentiated spermatogonia were established from THY1+ cell populations isolated by magnetic activated cell sorting from testes of pup (P6–P8) or adult (3–4 months) Rosa26-LacZ hybrid mice as described previously (Kubota et al., 2004a; Oatley and Brinster, 2006). Cultures were maintained in either conventional or GO conditions and mitotically inactivated STO feeders. The conventional condition included chemically defined serum-free medium (mSFM) supplemented with a growth factor cocktail of GDNF and FGF2, a mixture of free fatty acids, and an atmosphere of 5% CO2 and 21% O2 as described by Kubota et al. (2004b). The GO condition was identical except for elimination of the free fatty acid supplement and reduction of O2 tension to 10%. For all cultures, medium was replaced every 2 days and cells were subcultured onto fresh feeders every 6–7 days.

**Transplantation Analyses**

Evaluation of SSC content and their regenerative integrity in primary undifferentiated spermatogonial cultures at various time points was conducted using transplantation analysis. In brief, cultured cell populations were dissociated into single-cell suspensions by digestion with trypsin/EDTA solution, a portion (1 × 10^5 cells) was removed and suspended in mSFM at a concentration of 1 × 10^6 cells/mL, and the remaining cells were replated for continued culture. For transplantation into busulfan-treated recipients, ~10 μL of cell suspension (~1 × 10^4 cells) was microinjected into the seminiferous tubules of each testis as described previously (Helsel and Oatley, 2017; Oatley and Brinster, 2006). For evaluation of incomplete colonies, W/Wv recipients were transplanted with cells from 2-month cultures suspended at a concentration of 1 × 10^7 cells/mL and ~5 μL (~1 × 10^5 cells) was microinjected per testis. Recipient testes were evaluated for donor-derived colonies either 2 months (busulfan-treated) or 3 months (W/Wv) after transplantation by staining with β-galactosidase as described previously (Helsel and Oatley, 2017; Oatley and Brinster, 2006).

**Testis Histology and Immunostaining**

Testes were fixed in 4% paraformaldehyde and embedded in paraffin, and serial cross-sections of 5–7 μm thickness were made through the entire tissue and mounted on glass slides. Histological
analysis was performed by H&E staining after deparaffinization. For immunostaining analyses, deparaffinized cross-sections were incubated overnight at 4°C with primary antibodies against TRA98 (rat monoclonal, Abcam, cat. #ab82527) and LIN28 (goat polyclonal, R&D Systems, cat. #AF3757), diluted 1:200. After extensive washing in PBS, sections were incubated with secondary antibodies at a dilution of 1:1,000 for 4 hr at room temperature. All cross-sections were visualized with an inverted fluorescent microscope and images were captured with a DP72 digital camera using CellSense software (Olympus).

**Lactate Assay**
To determine whether different culture conditions altered glycolytic activity in the spermatogonia, we measured lactate concentration of culture medium. In brief, a standard number of cells was seeded per culture well and maintained in conventional or GO conditions for 1 week during which time the culture medium was replaced at 48-hr intervals. After the last interval, lactate concentration of the medium was measured using a commercial ELISA (EnzyChrom Glycolysis Assay, BioAssay Systems, product #EGL1-100). Because the spermatogonia were co-cultured with STO feeders, we first assessed whether the different culture conditions affected their glycolytic activity. Outcomes revealed no difference between conventional and GO conditions (Figure S2), thus eliminating effects on the feeder as a confounding factor of the measurements made for the cultured spermatogonia.

**Quantification and Statistical Analysis**
All quantitative data are presented as the mean ± SEM, and n values are described in the text of the Results section as well as the figure legends. Significant differences between means were determined statistically using the one-way ANOVA function of GraphPad Prism 6 software.

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes two figures and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2017.03.004.

**AUTHOR CONTRIBUTIONS**
A.R.H.: experimental design, collection of data, analysis of data, and manuscript writing; M.J.O.: experimental design and collection of data. J.M.O.: experimental design, collection of data, analysis of data, manuscript writing, financial support, and final approval of manuscript.

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