Indirect Effects of Wnt3a/β-Catenin Signalling Support Mouse Spermatogonial Stem Cells In Vitro

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Abstract

Proper regulation of spermatogonial stem cells (SSCs) is crucial for sustaining steady-state spermatogenesis. Previous work has identified several paracrine factors involved in this regulation, in particular, glial cell line-derived neurotrophic factor and fibroblast growth factor 2, which promote long-term SSC self-renewal. Using a SSC culture system, we have recently reported that Wnt5a promotes SSC self-renewal through a β-catenin-independent Wnt mechanism whereas the β-catenin-dependent Wnt pathway is not active in SSCs. In contrast, another study has reported that Wnt3a promotes SSC self-renewal through the β-catenin-dependent pathway, as it can stimulate the proliferation of a spermatogonia cell line. To reconcile these two contradictory reports, we assessed Wnt3a effects on SSCs and progenitor cells, rather than a cell line, in vitro. We observed that Wnt3a induced β-catenin-dependent signalling in a large subset of germ cells and increased SSC numbers. However, further investigation revealed that cell populations with greater β-catenin-signalling activity contained fewer SSCs. The increased maintenance of SSCs by Wnt3a coincided with more active cell cycling and the formation of germ cell aggregates, or communities, under feeder-free conditions. Therefore, the results of this study suggest that Wnt3a selectively stimulates proliferation of progenitors that are committed to differentiation or are in the process of exiting the SSC state, leading to enhanced formation of germ cell communities, which indirectly support SSCs and act as an in vitro niche.

Introduction

Spermatogonial stem cells (SSCs) are the foundation of life-long spermatogenesis. SSCs have the unique ability to maintain the stem cell pool through self-renewing divisions as well as to generate daughter cells committed to differentiation thereby producing mature sperm. This fate decision is believed to be tightly regulated during steady-state spermatogenesis and occurs in a specialized microenvironment, referred to as the niche, which supports SSCs [1]. The concept of a niche is well demonstrated in the Drosophila testis where somatic cells, hub cells, are located at the distal tip of the testis and are responsible for maintaining the stemness of germ-line stem cells through direct contact [2]. Migration away from contact with hub cells leads to differentiation toward sperm.

Interestingly, it has been shown that differentiated germ cells can regain stem cell activity upon homing back to hub cells suggesting the inductive nature of the Drosophila testis niche [3].

Such a defined niche has not been characterized in mammalian testes. Rather, SSCs reside along the basement membrane of the seminiferous tubule in close contact with Sertoli cells, the supporting somatic cells [1]. A great deal of work has been performed to identify how Sertoli cells support SSCs. To date, a handful of factors derived from testicular somatic cells, such as Sertoli cells, peritubular myoid cells, and Leydig cells, have been demonstrated to influence SSC renewal, in particular glial cell-line derived neurotrophic factor (GDNF), fibroblast growth factor 2 (FGF2), colony stimulating factor 1, and Wnt5a [4,5,6,7]. In contrast, knowledge of factors associated with SSC commitment to differentiation is limited. In addition, SSCs are not only in close contact with Sertoli cells but also with other types of spermatogonia in the niche. Therefore, the possibility exists that non-stem spermatogonia can communicate with SSCs and contribute to the regulation of SSC activity. However, this possibility has not been investigated.

Although mechanisms that control SSC fate are not completely understood, SSCs can be expanded in vitro under well defined conditions. In the presence of GDNF and FGF2, SSCs proliferate in vitro and form distinct accumulations of SSCs and daughter spermatogonia, which we term “clusters” [4,8,9]. Interestingly, previous work has shown that SSCs are a minority population among cluster cells, and the reported percentages of SSCs in clusters are relatively constant (1–3%) across several studies [5,8,9]. These observations raise a possibility that clusters constitute a society of male germ cells in vitro in which cells communicate with one another, thereby regulating the proportion of SSCs in the community [10].

In a previous study, we reported the expression of various Wnts in mouse testes and feeder cells used in SSC cultures [7]. Wnts are a family of lipid-modified, secreted glycoproteins with diverse functions in embryogenesis, tumorigenesis, as well as stem cell proliferation and differentiation [11,12]. In general, Wnt proteins can activate two classes of signalling cascades. The better characterized canonical (β-catenin) pathway involves Wnt stimula-
tion with its receptor Frizzled and co-receptor low density lipoprotein-related protein (LRP) 5/6, ultimately resulting in interaction between β-catenin and members of the T-cell factor/Lymphoid enhancer factor (TCF/LEF) transcription factor family in the nucleus and modulation of target gene transcription. In contrast, non-canonical pathways involve a wide host of mediators that do not act through β-catenin.

Previous studies have demonstrated the importance of Wnt signalling in the niches of various adult stem cell types [13]. In vitro, β-catenin signalling promotes the expansion of phenotypically-defined hematopoietic stem cells (HSCs), neural stem cells (NSCs), and intestinal stem cells (ISCs) [14,15,16]. However, further studies show that HSCs can self-renew in the absence of β-catenin [17] and while constitutive β-catenin-signalling can expand HSCs in vitro, these cells do not retain the functional ability to differentiate into blood lineages [18]. Moreover, β-catenin signalling appears to be dispensable for NSCs and rather supports the adhesion and survival of progenitor cells in neurospheres [19]. Stem cells are defined functionally; therefore, these studies serve to highlight the importance of assessing pathway activation effects on stem cell activity in a functional manner.

Recently, we identified Wnt5a as a novel factor, expressed by feeder cells and Sertoli cells, that supports SSC renewal [7]. Using the functional transplantation assay for SSCs, we demonstrated that Wnt5a acts through a non-canonical mechanism and promotes SSC survival and self-renewal. Furthermore, the β-catenin pathway was not activated in SSCs and spermatogonia with active β-catenin signalling had committed to differentiation. In contrast, a study by Golestanian et al. [20] has shown that Wnt3a stimulates the β-catenin pathway and leads to proliferation of an SV40-transformed spermatogonia cell line. However, the identity of this cell line as SSCs has not been examined functionally; i.e., the ability of these cells to regenerate and maintain spermatogenesis has not been demonstrated.

In an attempt to reconcile these two contrasting results and to better understand the role of Wnt/β-catenin-signalling in controlling SSCs and progenitor spermatogonia, we set out to functionally assess the effect of Wnt3a using SSC culture. We hypothesized that in accordance with our previous work Wnt3a would activate β-catenin signalling and reduce SSC numbers in vitro. However, Wnt3a surprisingly led to a significant increase in SSC numbers. We also observed that Wnt3a stimulated the β-catenin pathway in a large subset of cluster cells. These results appeared to contradict the findings of our previous study. However, further investigation demonstrated that although Wnt3a stimulated β-catenin signalling in a large population of germ cells, this population contains markedly reduced numbers of SSCs suggesting that the increase in SSC numbers occurred in cluster cells in which the β-catenin pathway was not activated. In addition, Wnt3a-induced increase in SSC numbers correlated with the increased formation of cell aggregations and cell-cell associations. Therefore, our study suggests that Wnt3a may regulate SSC activity indirectly by acting on committed daughter cells in vitro.

**Results**

**Wnt3a Increases SSC Numbers in vitro**

A recent study has described the ability of Wnt3a to activate β-catenin signalling and stimulate proliferation in an SV40-transformed spermatogonia cell line [20]. This cell line is immunophenotypically similar to SSCs but has not been functionally tested as SSCs using spermatogonial transplantation. Therefore, whether Wnt3a affects SSCs in a similar manner to this cell line is unknown. To address this, we initially used an in vitro culture system in which SSCs can be expanded. This culture system uses the growth factors GDNF and FGF2 and a layer of mitotically inactivated mouse embryonic fibroblasts (STO cells) as feeder cells [4,9]. Under these conditions, SSCs and their daughters can be expanded over a long period as distinct communities, termed “clusters” (Fig. 1A), of which SSCs comprise the minority population [5,9]. Recombinant Wnt3a was added to B6ROSA clusters for one week and clusters developed similarly under both conditions (Fig. 1A). We transplanted treated clusters into the testes of infertile recipient mice to assess Wnt3a effects on SSC activity. Using this spermatogonial transplantation technique, SSCs normally hidden among the cells of a cluster can be unequivocally detected by their ability to regenerate spermatogenesis. Two months following transplantation, recipient mouse testes were analyzed to count colonies of regenerated spermatogenesis. We observed that Wnt3a led to a 1.4-fold increase in numbers of functional SSCs, but this increase was deemed to be not significantly different (Fig. 1B), leaving the result of Wnt3a on SSC activity somewhat ambiguous.

Previously, we reported the expression of other Wnt molecules by our STO feeder cells. To circumvent potential indirect effects via feeder cells, cluster cells were removed from feeder cells through gentle pipetting and cultured under feeder-less conditions on Matrigel-coated plates for a short duration (4 days) with growth factors and in the presence or absence of Wnt3a. Since our STO feeder cells are critical for cluster formation and SSC proliferation, clusters do not form under these conditions. Following treatment, cluster cells were recovered from feeder-less conditions and cultured back onto feeder cells to induce cluster formation. The number of clusters that form in this cluster-formation assay correlates to functional SSC numbers, thereby providing a short-term method to quantify SSCs [8]. Interestingly, we observed a dramatic dose-dependent increase in cluster-formation ability with the addition of Wnt3a (Fig. 1C). To verify this Wnt3a effect on functional SSCs, treated cluster cells were subjected to spermatogonial transplantation. We confirmed that the addition of Wnt3a led to a significant 2.7-fold increase in colony numbers (Fig. 1D), demonstrating that Wnt3a promotes SSC activity in vitro. Finally, we added the β-catenin signalling-specific inhibitor Dickkopf1 (DKK1) to Wnt3a-treated cultures and observed a significant decrease in colony numbers (Fig. 1E). Thus, these results indicate that the effects of Wnt3a on SSC activity are mediated by the β-catenin pathway.

**Wnt3a Activates β-catenin Signalling in a Subset of Cluster Cells and Reduces SSC Activity in these Cells**

Previously, we demonstrated that Wnt3a supported SSCs through a non-canonical mechanism, whereas Wnt3a has been reported to act primarily through β-catenin signalling [7]. Immunofluorescent staining for the Wnt co-receptor LRP5/6 showed that all cluster cells express this protein (Fig. 2A). LRP5/6, in conjunction with the Wnt receptor, Frizzled, is necessary to transduce the Wnt/β-catenin signal. Frizzled protein expression has been reported on cluster cells [7]; therefore, all cluster cells appear able to signal through the β-catenin pathway. To confirm if Wnt3a indeed activates the β-catenin signalling pathway in cluster cells, we used established cluster cells derived from transgenic reporter mice (TCF/LEF-lacZ mice). These mice carry the lacZ reporter gene downstream of TCF/LEF binding sites, allowing faithful monitoring of β-catenin signalling activation [21,22]. TCF/LEF-lacZ cluster cells were treated with Wnt3a on Matrigel, and the presence of β-catenin signalling cells were visually quantified after 4 days. On day 1, we detected a dramatic increase in the percentage of β-catenin signalling cells compared to
untreated control cluster cells (Wnt3a vs. untreated; 79.0±3.6 vs. 1.8±0.2%) (Fig. 2B, C). Interestingly, the levels of β-catenin signalling activation varied in these cells with a gradient from moderate to robust lacZ expression (Fig. 2B, C). By day 4 of culture, we noted that this proportion of TCF/LEF-\(\text{lacZ}\) dim and high cells remained constant. We did not detect a significant change in total cell numbers upon Wnt3a stimulation under this feeder-free condition (Fig. 2D).

Previously, we demonstrated that global β-catenin pathway activation in all cluster cells using lithium chloride led to a loss in SSC activity suggesting that pathway activation may be associated with differentiation [7]. We therefore asked whether cluster cells stimulated by Wnt3a (i.e. an extracellular Wnt ligand vs. a global activator such as lithium) similarly lost SSC activity. To this end, TCF/LEF-\(\text{lacZ}\) cluster cells were cultured feeder-free in the presence of Wnt3a. Four days later, these treated cluster cells were recovered and reacted with a fluorescent vital β-galactosidase substrate (FDG) and then separated via FACS according to staining levels (Fig. 2E, F). Flow cytometric histograms of FDG activity were gated into three fractions, TCF/LEF-\(\text{lacZ}\) signalling negative (42.8±4.6%, Fraction II, Fig. 2F), dim (38.4±1.7%, Fraction III), and high expressing (18.7±3.4%, Fraction IV). The three fractions were isolated and separately transplanted into recipient testes to assess the SSC activity of each population. We observed that TCF/LEF-\(\text{lacZ}\) negative cells were highly enriched in functional SSCs (181.5±30.4 functional SSCs per 10^6 cells), while β-catenin signalling cells had minimal SSC activity, similar to our previous observation (Fig. 2G) [7]. The TCF/LEF-\(\text{lacZ}^\text{dim}\) population was composed of ~50 functional SSCs per 10^6 cells, while TCF/LEF-\(\text{lacZ}^\text{high}\) had ~18 functional SSCs per 10^6 cells showing a trend of declining SSC frequency with an increase in β-catenin signalling intensity. Additionally, we performed quantitative PCR to examine the expression levels of markers for SSCs and differentiating spermatogonia (Fig. S1). We observed a trend showing decreased expression of Plzf in TCF/LEF-\(\text{lacZ}^\text{high}\) cells and increased expression of Ngn3 and c-Kit in TCF/LEF-\(\text{lacZ}^\text{dim}\), however no significance was detected. Therefore, these results collectively demonstrate that Wnt3a stimulates the β-catenin pathway in a subset of cluster cells but the population of functional SSCs is significantly diminished as β-catenin signalling is activated.

Wnt3a Stimulates Spermatogonial Progenitors

Since a previous study reported that proliferation of a spermatogonia cell line was stimulated by Wnt3a [20], we analyzed cell cycle profiles of cluster cells after Wnt3a treatment using propidium iodide staining and flow cytometry. Cluster cells were treated with or without Wnt3a overnight, under feeder-free conditions, and profiles were assessed the next day. We observed that most cluster cells are not actively cycling (G0/G1 phase) (Fig. 3A). However, following Wnt3a treatment we observed a significant 3-fold increase in the percentage of actively cycling cells (in S, G2, & M phase). We next asked whether active cycling is induced equally in both β-catenin signalling and non-signalling
cells. To this end, TCF/LEF-lacZ clusters were stimulated with Wnt3a and separated via flow cytometry. Cell cycle profile analysis of each fraction showed that the fraction of actively cycling cells was markedly higher in β-catenin signalling cells than in non-signalling cells (negative: 6.7±1.3%, dim: 21.9±1.9%, high: 21.8±1.4%), (Fig. 3B). These results indicate that Wnt3a activates the β-catenin pathway in a select population of cluster cells and drives their cell cycle.

Over the course of our short-term feeder-free culture, we observed that “cluster-like” accumulations formed in the presence of Wnt3a, while in its absence most cells remained as singles or doublets (Fig. 4). Therefore, to quantify whether Wnt3a led to an increase in the frequency of directly-contacted, aggregating cells, we employed CellProfiler image analysis software [Broad Institute] [26] and monitored the daily growth of B6GFP cluster cells in the presence of Wnt3a, comparing them to untreated controls. We utilized an algorithm that identified individual cells based on GFP expression and the presence of a DNA dye. This algorithm then measured how many adjacent, neighboring cells each cell directly contacts, thereby identifying the frequency of cell aggregation. The results showed that in the absence of Wnt3a, most cells remain with 0–1 connected cells (i.e. singles or doublets) throughout the course of culture (Fig. 5A, B, higher magnification image is found in Fig. S2). In contrast, Wnt3a-treated cells started as singles or doublets at day 1 of culture, similar to controls. However, the frequency of singles/doublets declined throughout the course of culture until most cells had at least 1–2 neighboring cells, while only ~14% of cells are classified as singles by day 4 (Fig. 5C, D). Furthermore, Wnt3a-treated cultures showed 10% of cells with 4 or more neighboring cells by day 4, which represents a significant increase compared to untreated controls where only 5% of cells have 4 or more neighbors. Therefore, these results show that Wnt3a stimulation leads to the formation of cluster-like cell aggregations under feeder-free conditions.

Finally, we attempted to determine if Wnt3a leads to SSC expansion over the course of 4 day culture, under feeder-free conditions. Using the cluster-formation assay, we compared the numbers of SSCs at the beginning (day 0) to the end (day 4) of
feeder-free culture with or without Wnt3a. From this comparison we can determine if SSC numbers increase, decrease, or are simply maintained. From day 0 to day 4, SSC numbers increased 1.3-fold with Wnt3a, which was deemed not significant (Fig. 5E). In the absence of Wnt3a cluster-forming cells decreased dramatically. Therefore, these results suggest that development of cell-cell associations supports the maintenance of SSC activity over the course of feeder-free culture.

**Discussion**

The results of this study indicate that Wnt3a can activate the β-catenin pathway in a subset of cluster cells but the majority of these cells are not SSCs, which is consistent with our previous study [7]. Interestingly, Wnt3a treatment leads to an overall increase in SSC numbers. We observe that β-catenin pathway activation leads to increased cell cycle activity and more robust

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**Figure 3.** Wnt3a stimulates cluster cell cycling. (A) Percentage of non-cycling (G0/G1; open bars) and actively cycling (S/G2/M; filled bars) cells following Wnt3a stimulation. (B) Percentage of cycling cells in β-catenin signalling populations following Wnt3a stimulation. Representative flow cytometric histograms show β-catenin signalling positive cells are actively cycling after Wnt3a stimulation.

**Figure 4.** Wnt3a leads to increased “cluster-like” aggregations. Representative brightfield photomicrographs of cluster cells following control or Wnt3a treatment at each day of culture. At day 1 after seeding, cluster cells are arranged predominantly as singles or doublets under both conditions. At day 2, most cells remain as singles and doublets in both conditions but longer chains are more easily observable (arrowheads; upper panels are higher magnification). In day 3 control cultures, most cells remain as singles but small clumps are occasionally observed (arrowhead). In Wnt3a-treated cultures, larger clumps are observable by day 3 (arrowheads). On day 4, large “cluster-like” cell aggregations are found throughout Wnt3a-treated cultures (arrowheads), while control cultures are mostly singles and infrequently show small cell accumulations or the occasional cell chain (arrowheads) but lack large cell accumulations. Upper panels show higher magnification images. Scale bar: 50 μm, upper panels: 12.5 μm.

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cell-cell association upon Wnt3a treatment, resulting in an increase in SSC maintenance. Thus, these results collectively suggest that Wnt3a increases SSC numbers indirectly through stimulating the formation of the cluster community, raising the possibility that the presence of committed cells might be important to maintain SSCs.

During steady-state spermatogenesis, SSCs are believed to represent a population of the most primitive A single spermatogonia. However, following injury or depletion it has been shown that more differentiated A paired or A aligned spermatogonia may revert to an SSC fate [27,28]. Accordingly, cluster cells expressing the differentiation marker c-kit are observed to retain SSC regenerative ability equal to their c-kit counterparts [29], implying that all cells within the cluster community may retain a level of SSC activity. We observed that the majority of functional SSCs were TCF/LEF-lacZ non-signalling cells and that SSC activity is lost as activation levels of β-catenin signalling increased (Fig. 2). Therefore, it is possible that cluster cells may be induced for gradual commitment to differentiation upon activation of this signalling pathway. These observations lead us to speculate that activation of β-catenin signalling may artificially force cells toward commitment. Thus, the experimental paradigm used in this study may offer a means to examine the factors mediating differentiation, in particular, to the level of differentiation where SSC commitment becomes irreversible. Analyzing global gene expression profiles of the β-catenin signalling and non-signalling populations may provide information about the mechanism of SSC commitment at the molecular level.

How might Wnt3a stimulation support SSC maintenance? We found that manipulation with Wnt3a led to the formation of germ cell aggregations that resemble clusters, potentially as a result of activated cell cycle in non-SSCs. Interestingly, we note that tissue specific stem cells, which can proliferate ex vivo, have not been maintained alone but are always accompanied by differentiated daughter cells, such as seen in neurospheres and mammospheres.
Materials and Methods

Donor Animals

Homozygous TCF/LEF-lacZ mice (from Dr. D. Dufort, McGill University) are on a CD-1 genetic background and carry the lacZ reporter gene driven by β-catenin-TCF/LEF responsive elements [21,22]. B6ROSA mice are F1 hybrids of C57BL/6 (B6) and ROSA26 mice, which express the lacZ gene ubiquitous in virtually all cell types [23]. B6GFP mice (C57BL/6-Tg(CAG-EGFP)1Osb/J; The Jackson Laboratory) express GFP ubiquitously. Animal procedures were approved by the Animal Care and Use Committee of McGill University.

Recipient Animals and Transplantation

Spermatogonial transplantation was performed and recipients were prepared as described previously [7]. Recipient mice for B6ROSA cells were 129/SvEv × B6 F1 hybrids, and those for TCF/LEF-lacZ cells, Ncr nu/nu mice (Taconic). Recipient animals were injected with busulphan i.p. (50 mg/kg for 129/SvEv × B6, 40 mg/kg for Ncr nu/nu) at 4–6 wk of age to eliminate endogenous spermatogenesis, at least 1 mo prior to transplantation. Donor cells were from cultures derived from 7–8 dpp prepubertal mouse testes. For transplantation, cultures were enzymatically digested to a single cell suspension and injected into the rete testis of recipient mice, to fill the seminiferous tubules. Recipient testes were analyzed for SSC quantification following staining with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) 2 months post-transplantation. Colony numbers of donor-derived spermatogenesis were visually counted [24] and expressed as colonies/105cells initially placed in culture.

Cell Culture

SSC cultures were established from immunomagnetic selected Thy1-positive testis cells from 7–8 days post partum (dpp) mice as described previously [8]. Cultures were maintained with a serum-free MEMα-based medium with supplements on a feeder layer of STO fibroblasts. Media were replenished every 3rd day and clusters were subcultured onto freshly prepared STO feeder cells every 6–7 days. Cultures were maintained with “growth factors” consisting of GDNF (20 ng/ml), GFRα1 (75 ng/ml), and FGF2 (1 ng/ml) [4,8]. Experiments were conducted using established cluster cells (>5 passages), removed from STO feeder cells using gentle pipetting, which results in an isolation of cluster cells at more than 90% purity [7]. Subsequently, clusters were digested to single cells following treatment with 0.05% trypsin-EDTA. For short-term feeder-free cultures, culture plates were coated with Matrigel (BD Bioscience), diluted 1:2 in serum-free culture media, and incubated overnight, and visually counted using a hemocytometer.

Cluster formation Analysis

As a short-term semi-quantitative assay for SSC activity, it has been determined that cluster number correlates with functional SSC number [8]. Hence, cluster cells exposed to experimental conditions were subcultured onto STO feeder cells in the presence of growth factors to induce cluster formation. Media were replenished 3 days
after initial seeding and by day 6 cluster numbers were ready for quantification. Cluster numbers were determined in one of the following two ways. B6ROSA-derived clusters were reacted with X-gal overnight and cluster numbers were counted visually under a microscope. B6GFP-derived cluster numbers were acquired automatically using an ImageXpress Micro imaging system (Molecular Devices) as described previously [25]. Three experiments were performed for all cluster formation analyses and the average of at least two wells was recorded for each group per experiment. Cluster numbers were normalized to $10^6$ cells placed in culture.

**Flow Cytometric Analysis and Sorting**

To isolate β-catenin-signalling cell populations following Wnt3a stimulation, TCF/LEF-lacZ clusters were reacted with 500 µM fluorescein di-β-D-galactopyranoside (FDG, Marker Gene Technologies) and sorted as described previously [7]. Experimental gates were established using control cells: B6ROSA (positive) and B6 (negative) cluster cells. For cell cycle analyses, B6ROSA cluster cells were fixed in 70% ethanol at −20°C overnight followed by incubation with 40 µg/ml propidium iodide (PI) and 100 µg/ml RNase at room temperature. Data were acquired on a FACSscan or Accuri C6 Cytometer (Becton Dickinson), from three experiments with at least 10,000 events collected per sample.

**Cell Profiler Analysis**

To quantify Wnt3a effects on germ cell clustering, B6GFP cluster cells were cultured feeder-free in a 96-well plate at $5 \times 10^4$ cell/cm² from 1 to 4 days in the presence or absence of Wnt3a. The time of cell seeding was deemed Day 0. Cultures were terminated each day from days 1 to 4 and, at each day, were reacted with a DNA dye (DAPI), to assist in the identification of cells. Thirteen representative fluorescent photomicrographs were taken from various points randomly in each culture well. For neighboring analyses we used the CellProfiler Software Platform (Broad Institute) [26]. To this end, a pipeline was developed that identified individual cells based on their shape and expression of neighbor. Analyses we used the CellProfiler Software Platform (Broad Institute) [26]. To this end, a pipeline was developed that identified individual cells based on their shape and expression of c-Kit are observed in the TCF/LEF-lacZ-signalling populations.

**Immunofluorescent Staining**

B6ROSA cluster cells were cultured on Matrigel as above with Wnt3a. The time of cell seeding was deemed Day 0. Cultures were terminated each day from days 1 to 4 and, at each day, were reacted with a DNA dye (DAPI), to assist in the identification of cells. Thirteen representative fluorescent photomicrographs were taken from various points randomly in each culture well. For neighboring analyses we used the CellProfiler Software Platform (Broad Institute) [26]. To this end, a pipeline was developed that identified individual cells based on their shape and expression of c-Kit are observed in the TCF/LEF-lacZ-signalling populations.

**Quantitative PCR**

Total RNA was extracted using the PicoPure RNA Isolation Kit (Applied Biosystems). Complementary DNA was synthesized using Superscript III Reverse Transcriptase (Invitrogen) with random hexamers. Quantitative PCR was performed with the QuantiTect SYBR Green PCR Kit (Qiagen) on a Rotorgene 6000 (Corbett Research) with the program: 94°C for 15 min followed by 40 cycles of 94°C for 15 sec/51–60°C for 30 sec/72°C for 35 sec. Primer sequences were the following: c-Kit-F: TGGGAATTTCCAGAAGAACAG, c-Kit-R: AAATGGGCCAGTCGTTTGAG; Nanos2-F: TGAGGTACCTGTCACCACA, Nanos2-R: GGATCCGTGAAAGGCACAGAAA; Ngn3-F: CTTCAATGGGAGAATTCCTG, Ngn3-R: TTCCCACTAGCACCCACAC; Plzf-F: GCAGCTATAATTGGCAATGGAG, Plzf-R: TCCTGAGTGTGCTCCTATCC.

**Statistics**

Data were expressed as mean ± SEM. Numbers of clusters in vitro and SSCs detected with spermatogonial transplantation were indicated as those per $10^6$ cells placed in culture, unless specified otherwise. Significance was determined using ANOVA followed by Fisher’s Test for Least Significant Difference. $p<0.05$ determined significance.

**Supporting Information**

**Figure S1 Expression of spermatogonia markers in TCF/LEF-lacZ signalling populations.** Quantitative PCR on TCF/LEF-lacZ-negative cells (open bars), TCF/LEF-lacZlim cells (shaded bars), and TCF/LEF-lacZhigh cells (filled bars). TCF/LEF-lacZhigh cells show lower expression of Plzf. Greater expression of the differentiating spermatogonial markers Ngn3 and c-kit are observed in the TCF/LEF-lacZlim/high cell fractions. No significant difference was observed for any marker. (EPS)

**Figure S2 CellProfiler demarcates individual cells in an aggregation.** Representative fluorescent photomicrographs of B6GFP cluster cells following control (top panels) or Wnt3a treatment (bottom panels) after 4 days. (A–B) Fluorescent images showing DAPI and GFP expression in cluster cultures. Large and more densely growing “cluster-like” cell aggregations are found in Wnt3a-treated cultures compared to untreated controls. (C) Representative images of individual cell clusters. CellProfiler identifies cells based on the co-expression of DAPI and GFP and demarcates these individual cells using red outlines. (D) CellProfiler image analysis identifying individual cells. Cells are colored green if they have at least one directly-adjacent neighbor cell. The color gradient labels individual cells according to the number of directly-connected neighboring cells. Note the difference in the neighboring cell numbers indicated by the color gradient between control and Wnt3a groups. Scale bar: 48 µm. (EPS)

**Author Contributions**

Conceived and designed the experiments: JY MN. Performed the experiments: JY XZ. Analyzed the data: JY MN. Wrote the paper: JY MN.

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