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Wnt signaling promotes proliferation and stemness regulation of spermatogonial stem/progenitor cells

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Abstract

Spermatogonial stem cells (SSCs) self-renew throughout life to produce progenitor cells that are able to differentiate into spermatozoa. However, the mechanisms underlying the cell fate determination between self-renewal and differentiation have not yet been delineated. Culture conditions and growth factors essential for self-renewal and proliferation of mouse SSCs have been investigated, but no information is available related to growth factors that affect fate determination of human spermatogonia. Wnts form a large family of secreted glycoproteins, the members of which are involved in cell proliferation, differentiation, organogenesis, and cell migration. Here, we show that Wnts and their receptors Fzs are expressed in mouse spermatogonia and in the C18-4 SSC line. We demonstrate that WNT3A induces cell proliferation, morphological changes, and cell migration in C18-4 cells. Furthermore, we show that β-catenin is activated during testis development in 21-day-old mice. In addition, our study demonstrates that WNT3A sustained adult human embryonic stem (ES)-like cells derived from human germ cells in an undifferentiated stage, expressing essential human ES cell transcription factors. These results demonstrate for the first time that Wnt/β-catenin pathways, especially WNT3A, may play an important role in the regulation of mouse and human spermatogonia.

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Introduction

The testis structure is established as the primordial germ cells (PGCs) proliferate and differentiate into gonocytes, which are mitotically quiescent in mice until approximately day 2 after birth. Gonocytes reach the basement membrane of the seminiferous tubules or remain near the lumen and degenerate. Those that survive differentiate into type A spermatogonia, including spermatogonial stem cells (SSCs; de Rooij 2001). SSCs have the capacity to self-renew and to generate a large number of sperm (Dym 1994, de Rooij 1998). They are immortal cells since sperm transmit the male genome from generation to generation. The SSCs reside on the basement membrane of the seminiferous tubules and are closely related to the Sertoli cells that provide the growth factors and the extracellular signals essential for their self-renewal or differentiation. The maintenance of the SSC and thus normal spermatogenesis are meticulously regulated by intrinsic gene expression within the stem cell and the stem cell niche formed by Sertoli cells, the basement membrane, and possibly blood vessels (Brinster 2002, Yoshida *et al*. 2007). It has been demonstrated that glial cell line-derived neurotrophic factor (GDNF), GDNF-family receptor α-1, and fibroblast growth factor 2 (FGF2) are essential for replication and expansion of mouse SSCs (Meng *et al*. 2000, Kubota *et al*. 2004*b*, Hofmann *et al*. 2005*b*, He *et al*. 2007, 2008). However, the role of other growth factors and signaling pathways remains unclear. Furthermore, to date, there is no study related to the growth factors and signaling pathways indispensable for the culture and fate of human SSCs.

The Wnt family of protein ligands affects diverse processes including embryogenesis, generation of cell polarity, and the specification of cell fate (Logan & Nusse 2004). This pathway enables cells to communicate with each other and to coordinate various cellular processes, such as cell proliferation, differentiation, survival, apoptosis, migration, and cell fate. The extracellular Wnt signal stimulates numerous signal transduction cascades. To date, 19 members of the Wnt family have been identified in mammals and, depending on the Wnt, can signal through essentially two main classes of Wnt-signaling

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pathways, the canonical and non-canonical Wnt pathways (Du *et al*. 1995). The canonical pathway regulates the target gene expression in the nucleus controlling proliferation (Cadigan & Liu 2006), and the non-canonical pathways regulate many other aspects of cell biology such as cell motility and morphology (Kohn & Moon 2005). A hallmark of the canonical Wnt pathway is the nuclear localization of β-catenin (Brown 2005). In the absence of Wnt signaling, β-catenin is phosphorylated by glycogen synthase kinase-3, in association with axin and adenomatous polyposis coli, which targets β-catenin for ubiquitinylation and subsequent degradation by proteasomes (Cadigan & Nusse 1997). Active Wnt signaling disrupts this complex and induces β-catenin stabilization and nuclear localization (Willert & Jones 2006). The expression and possible roles of Wnts were reported in developing and adult testis (Katoh 2001, Jeays-Ward *et al*. 2003, Jordan *et al*. 2003, Boyer *et al*. 2008); however, to date, there is no evidence for the role of Wnts and especially WNT3A in SSC/progenitor cell regulation and proliferation.

The aim of this study was to investigate the expression of WNT3A in a mouse model of SSCs and to shed light on the role of WNT3A-mediated canonical and non-canonical pathways on SSC/progenitor cell proliferation and self-renewal. We also report that embryonic stem (ES)-like cells derived from human germ cells, presumably from SSC/progenitor cells, remain in an undifferentiated state in response to Wnt.

Results

Expression of Wnts and Frizzled in mouse spermatogonia and in a mouse SSC line (C18-4)

We have established a mouse SSC line (C18-4 cells) that presents many characteristics of mouse SSCs (Hofmann *et al*. 2005*a*, 2005*b*, Braydich-Stolle *et al*. 2007, He *et al*. 2007, 2008). The C18-4 cell line was used for RNA extraction and RT-PCR analysis. Specific primers for different Wnts have been used to perform RT-PCR. C18-4 cells showed expression of *Wnt2*, *Wnt3*, *Wnt3a*, *Wnt7b*, and *Wnt8a* among the ten different Wnts tested (Fig. 1A). The affinities between Frizzled (Fz) family members and Wnt ligands vary (Cadigan & Nusse 1997). We have detected the expression of *Fzd1*, *Fzd3*, *Fzd4*, and *Fzd5* receptors in C18-4 cells using specific primers for all ten different Fz receptors. The expression of *Fzd3* and *Fzd4* mRNA was higher than *Fzd1* in C18-4 cells (Fig. 1B). Consequently, we have analyzed the expression of *Fzd3* during development in mouse germ cells. The Sertoli cells produce GDNF that signals through GDNF family receptor alpha1 (GFRA1), which is expressed by SSCs (Buageaw *et al*. 2005). GFRA1 is used as a marker to isolate and enrich mouse SSCs by magnetic-activated cell sorting (MACS). In another

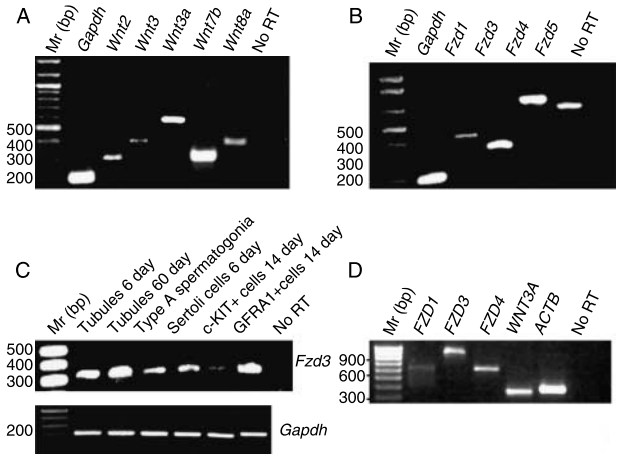

Figure 1 Expression of Wnts in C18-4 cells and in mouse and human isolated germ cells. (A) RT-PCR analysis of C18-4 cells with various Wnt primers. (B) RT-PCR analysis of C18-4 cells with different Frizzled primers. (C) RT-PCR analysis of Frizzled3 (*Fzd3*) with 6-, 14-, and 60-day-old mouse seminiferous tubules, type A spermatogonia isolated from 6-day-old mouse testis, c-KIT+ cells and GFRA1+ cells isolated form 14-day-old mouse testis. *Fzd3* expression was significantly higher in GFRA1+ cells compared to c-KIT+ cells. (D) RT-PCR analysis of *Wnt3a* and Fzs receptors in human germ cells isolated from adult human testis. *FZD3* and *FZD4* showed a high expression in these cells.

publication, it has been reported that c-KIT may be important for spermatogonial differentiation, but not for renewal (Ohta *et al*. 2000). We have isolated the seminiferous tubules from 6- and 60-day-old mice, Sertoli cells and type A spermatogonia from 6-day-old mice, SSCs from 14-day-old mice expressing GFRA1, and spermatogonia expressing c-KIT from 14-day-old mice. Figure 1C shows *Fzd3* expression in mouse germ cells. *Fzd3* was expressed in seminiferous tubules of 6- and 60-day-old mice. Six-day-old Sertoli cells and type A spermatogonia (6-day) also expressed *Fzd3*. Interestingly, the expression of *Fzd3* was significantly upregulated in the GFRA1-positive SSCs, compared with the c-KIT-positive spermatogonia suggesting that Wnt signaling may play an important role in SSC/progenitor cell regulation.

Although there are a few reports on the expression of the Fzs in rodent ovary and testis (Ricken *et al*. 2002, Hsieh *et al*. 2005, von Schalburg *et al*. 2006), there is no evidence showing the expression of Fzs in human germ cells. We have isolated human germ cells from adult testis as explained in the materials and methods section. After germ cell isolation and before culturing the cells, RNA was extracted from human germ cells containing SSCs/progenitor cells and RT-PCR analysis was performed for the expression of ten different Fzs and Wnts. Figure 1D shows the expression of *FZD1*, *FZD3*, *FZD4*, and *WNT3A* in adult human germ cells.

Wnt conditioned media stimulates β-catenin stabilization and signaling in C18-4 cells

On the basis of the observation that Wnts and Fzs were expressed in the C18-4 cell line, a follow-up study was designed to determine whether WNT3A or WNT10B conditioned media (CM) induce the canonical Wnt pathway by triggering the accumulation of free β-catenin in the cytoplasm. C18-4 cells at 70–80% confluency were treated with serum-free media, L-cell, WNT3A, or WNT10B CM for 3 h. Cell lysates were prepared and used for a pull-down assay using GST-E-cadherin to measure free β-catenin (Bafico *et al.* 1998). Figure 2A shows equal levels of total β-catenin protein expression in C18-4 cells treated with serum-free media, L-cell, WNT3A, or WNT10B CM. Free β-catenin levels, however, were significantly upregulated in C18-4 cells treated with WNT3A or WNT10B CM compared to the cells treated with serum-free media or L-cell CM. This observation demonstrates that WNT3A and WNT10B CM induce the stabilization and accumulation of free β-catenin in the cytoplasm in C18-4 cells. To test β-catenin activity, we transfected the C18-4 cells with a TCF7L2 (T-cell factor 7-like 2, formerly known as TCF4) promoter reporter construct (TOPFlash). It has been demonstrated that TCF7L2 interacts functionally with β-catenin to mediate Wnt signaling in vertebrates (Behrens *et al.* 1996, Molenaar *et al.* 1996, van de Wetering *et al.* 1997). Both WNT3A and WNT10B CM, as well as recombinant WNT3A, significantly increased the C18-4 cells' TOPFlash activity (Fig. 2B). WNT3A CM, WNT10B CM, and recombinant WNT3A did not activate an NFKB reporter construct showing that this activation by Wnts is specific to a β-catenin-responsive promoter.

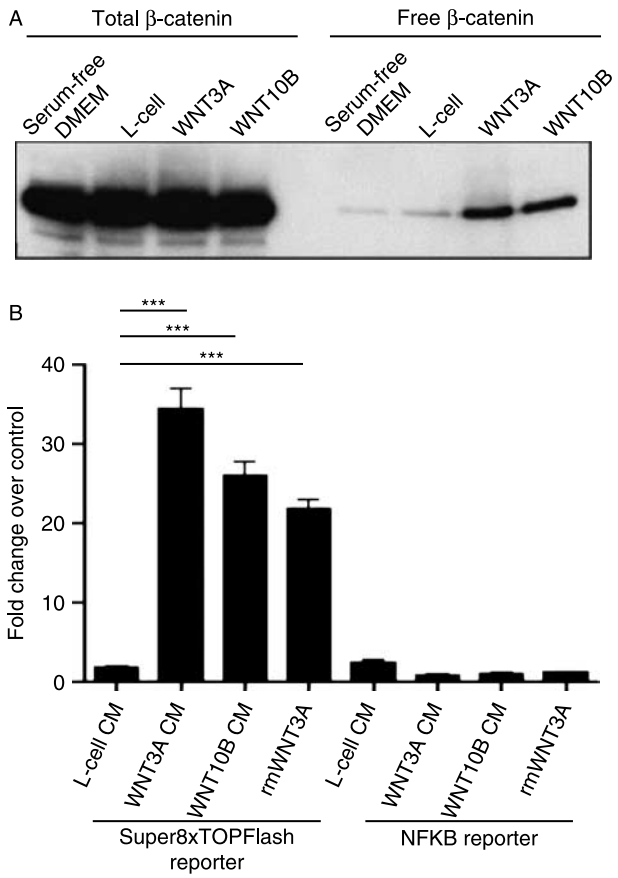

Figure 2 Treatment with Wnts causes an increase in β-catenin accumulation and signaling in C18-4 cells. (A) C18-4 cells were stimulated with various conditioned media for 3 h. Whole-cell lysates were analyzed for total and free β-catenin. Free β-catenin was measured by a GST pull down with a GST-E-cadherin construct. (B) Topflash assay. C18-4 cells were transfected with a Super 8×TOPFlash construct to measure active β-catenin signaling or an NFKB reporter construct used as a negative control. Cells were treated for 24 h with various conditioned media or 300 ng/ml of recombinant WNT3A. Columns represent the fold activation of the relative luciferase activity over the serum-free DMEM control. Relative luciferase activity was calculated by dividing the luciferase activity by the *Renilla* activity used as a transfection control. Error bars are the S.D.S (***$P<0.001$ using a two-tailed Student's *t*-test). Transfection assays were performed in triplicate.

WNT3A and WNT10B CM induce proliferation of C18-4 cells and freshly isolated spermatogonia

To verify whether Wnt signaling plays a role in self-renewal of C18-4 cells, we have performed a proliferation assay using a non-radioactive cell proliferation assay kit. Figure 3A shows the effect of Wnt CM on proliferation of C18-4 cells. The WNT3A and WNT10B CM induced respectively approximately fivefold and approximately fourfold increase in C18-4 cell number compared to the cells treated with L-cell. Treatment of C18-4 cells with recombinant WNT3A also induced cell proliferation (Fig. 3B). In addition, WNT3A and WNT10B CM induced cell proliferation in a primary culture of spermatogonia isolated from 6-day-old mice, resulting in an approximately eightfold increase in spermatogonial cell number compared to the cells treated with control L-cell media (Fig. 3C and D).

WNT3A and WNT10B CM induce morphological changes in C18-4 cells

Non-canonical Wnt-signaling pathways function in a β-catenin-independent manner to regulate tissue polarity and cell fate decisions (Kuhl *et al.* 2000, Peifer & Polakis 2000, Veeman *et al.* 2003). These non-canonical pathways have also been termed the Wnt/calcium and Wnt/MAPK8 pathways in vertebrates and the Wnt/planar cell polarity pathway in flies. We have investigated the effect of WNT3A and WNT10B CM on C18-4 cell-line morphology. After treating the cells with L-cell, WNT3A and WNT10B CM for 3 h, the cells were fixed and the actin filaments were stained by phalloidin–FITC to verify the cytoskeletal rearrangements. C18-4 cells treated with WNT3A CM and WNT10B CM and recombinant WNT3A had an elongated cell body with

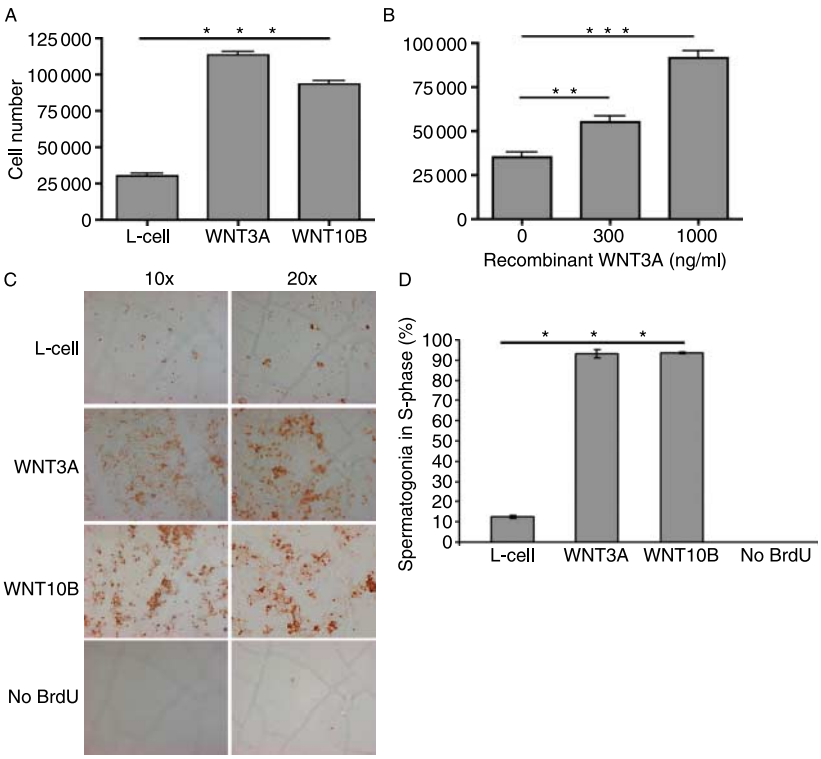

Figure 3 Treatment with Wnts can induce cell proliferation in C18-4 cells and in spermatogonial primary culture. (A) C18-4 cells were cultured in 96-well plates in the presence of L-cell CM, WNT3A CM, or WNT10B CM. After 48 h, the number of cells was analyzed using the CellTiter 96 non-radioactive cell proliferation assay kit. (B) C18-4 cells were assayed for cell proliferation after 48 h incubation in the presence of serum-free media containing 0, 300 ng/ml, and 1 μg/ml of recombinant WNT3A. (C and D) WNT3A and WNT10B promote the proliferation of murine spermatogonial cells *in vitro*. Results of immunostaining with anti-BrdU (C) and statistical analysis of the numbers of positive nuclei counted from three repeats out of the four treatments (D). The BrdU incorporation assays showed a significant increase (~80%) of cell proliferation in the cultures treated with either WNT3A or WNT10B, compared with the basic (L-cell) medium. (*$P<0.05$, **$P<0.01$, ***$P<0.001$ using a two-tailed Student's *t*-test).

several membrane extensions compared to the cells treated with L-cell CM (Fig. 4A). Figure 4B and C show the percentage of cells with membrane extensions that were equal to or longer than the length of the cell body. The percentage of cells with membrane extensions was significantly higher in the presence of WNT3A CM, WNT10B CM, and recombinant WNT3A compared with the controls (Fig. 4B). It has been reported that different isoforms of casein kinase I (CKI) modulate several components of Wnt signals, including generation of complete dorsal axes, stabilization of β-catenin, and induction of genes that are direct targets of Wnt signaling (Peters *et al*. 1999). We have used D4476, a specific and selective inhibitor of CKI-δ/ε, an isoform that controls DVL phosphorylation and non-canonical signaling (Bryja *et al*. 2007). CKI inhibitor D4476 completely abolished the WNT3A and WNT10B-induced membrane extensions in C18-4 cells (Fig. 4C), confirming that the morphological changes induced by WNT3A CM and WNT10B CM are owing to the non canonical Wnt/β-catenin pathway.

CKI inhibitor can prevent DVL phosphorylation but not β-catenin stabilization in C18-4 cells

In order to confirm that WNT3A CM- and WNT10B CM-induced morphology changes are owing to the non-canonical Wnt pathway, we have treated the C18-4 cells with WNT3A CM, WNT10B CM, and L-cell CM for 3 h in the presence and absence of D4476 (Rena *et al*. 2004). Figure 5A shows that WNT3A CM and WNT10B CM induced a significantly higher level of DVL2 and DVL3 phosphorylation, observed as a band shift, compared with the L-cell CM and that D4476 completely abolished WNT3A- and WNT10B-induced DVL phosphorylation. Interestingly, D4476 did not affect the activation of β-catenin by WNT3A CM and WNT10B CM (Fig. 5B). The results using the CM were verified by treating C18-4 cells with recombinant mouse WNT3A (Fig. 5C). Similar patterns were observed with the recombinant WNT3A-treated cells as those treated with CM. These observations further confirm that WNT3A- and WNT10B-induced morphology changes in C18-4 cells are owing to the non-canonical Wnt pathways.

WNT3A and WNT10B CM induce chemotaxis in C18-4 cells

Since WNT3A and WNT10B CM induced morphological changes in C18-4 cells, we wanted to determine whether WNT3A and WNT10B CM could also induce chemotaxis in C18-4 cells. The chemotaxis assay revealed that WNT3A and WNT10B CM induced cell motility in C18-4 cells compared to the control cells treated with L-cell CM (Fig. 6A). D4476 partially but significantly inhibited the Wnt-induced cell motility in C18-4 cells (Fig. 6B), suggesting that WNT3A and WNT10B induce cell motility in C18-4 cells through activation of non-canonical Wnt pathways.

β-catenin is activated during development in mouse spermatogonia

We investigated β-catenin expression in the 6-day-old mouse testis, when type A spermatogonia are the only cell type residing in the seminiferous tubules of the testis, versus 21-day-old mice testis, when spermatogonia A_s (single), A_{al} (aligned) and A_1–A_4, and spermatocytes are present but not sperm. Immunohistochemistry with a specific β-catenin antibody showed cytoplasmic and membrane staining for β-catenin in spermatogonia from the 6-days-old testes (Fig. 7A and C), whereas cells located on the basement membrane of the seminiferous tubules at 21 days showed nuclear β-catenin staining (Fig. 7B and D). As nuclear staining is a hallmark of an activated canonical Wnt pathway, these observations confirm a change in the functional state of β-catenin during development and further demonstrate the importance of the canonical Wnt pathway in SSC regulation.

WNT3A-CM maintain ES-like cells derived from human germ cells in an undifferentiated state

Recently, we have been able to isolate and culture adult human germ cells containing SSCs using human feeder-free ES cell culture media containing FGF2 and TGFB1. We were able to reprogram these cells into ES-like cells (Golestaneh *et al*. 2009) that formed colonies and expressed human ES cell-specific transcription factors and proteins. We have generated two cell lines that have been passaged for more than 15 passages (Golestaneh *et al*. 2009). RT-PCR analysis revealed that the ES-like colonies expressed markers of human ES cells such as *POU5F1*, *NANOG*, *ESG1*, *TDGF1*, and *SOX2*. Here, we use WNT3A CM and L-cell CM to culture human ES-like cells and to verify the effect of WNT3A on the maintenance of human ES-like cells in an undifferentiated stage. WNT3A CM maintained the ES-like cells in undifferentiated condition-forming colonies (Fig. 8A), whereas in the presence of L-cell CM the cells underwent differentiation (Fig. 8B). When we removed the FGF2 from the media, WNT3A CM was not sufficient to sustain long-term proliferation of the ES-like cells in feeder-free culture, data not shown. In addition, RT-PCR analysis of the human ES-like cells treated with WNT3A CM revealed the expression of human ES cell-specific transcription factors such as *POU5F1*, *NANOG*, *ESG1*, *TDGF1*, and *SOX2* (Fig. 8C), whereas these markers were absent in the human ES-like cells treated with L-cell CM (Fig. 8D).

Discussion

Wnt-signaling pathways play an important role in many developmental events (Kleber & Sommer 2004, Moon *et al*. 2004, Nelson & Nusse 2004). Recently, the Wnt canonical pathway has been implicated in stem cell self-renewal and cell fate determination (Hao *et al*. 2006, Ogawa *et al*. 2006, Cai *et al*. 2007, Nusse 2008). It has been demonstrated that Wnt/β-catenin activation expanded murine hematopoietic stem cell progenitors

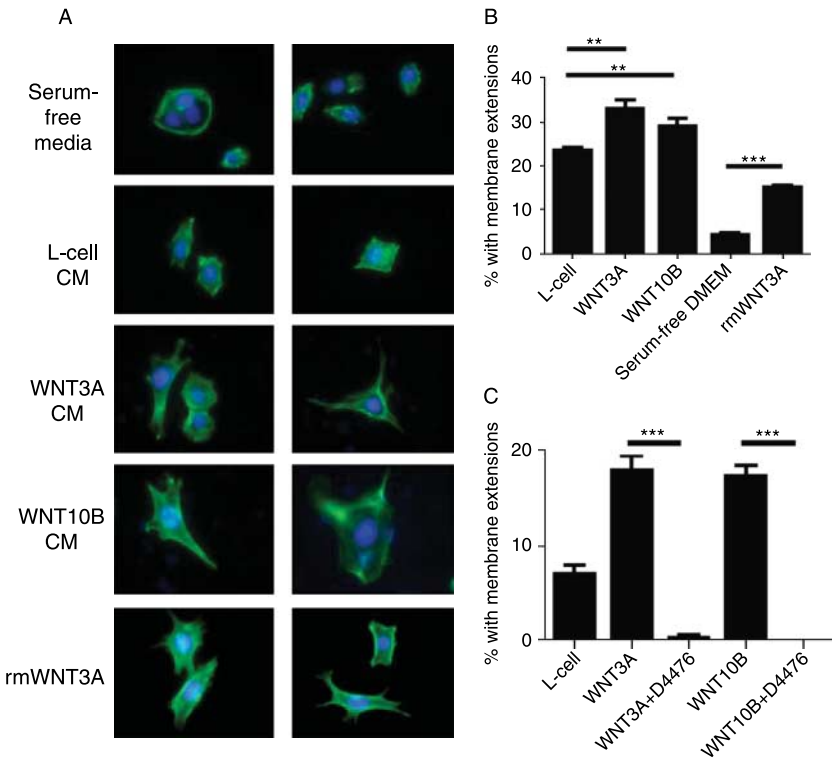

Figure 4 Treatment with Wnts causes a cell morphology change in C18-4 cells and can be inhibited with CKI inhibitor D4476. (A) C18-4 cells grown on cover slides were treated with (from top to bottom) serum-free media, L-cell CM, WNT3A CM, WNT10B CM, and recombinant WNT3A at 300 ng/ml in DMEM. Cells were fixed and stained with fluorescent-labeled phalloidin. Pictures were taken at 600× magnification. Two representative samples are shown for each treatment group. (B) Percentage of cells with membrane extensions. Experiments were performed in triplicate with 100 cells counted for each. Columns represent the means and error bars are the S.D.s (*$P<0.05$, **$P<0.01$, ***$P<0.001$ using a two-tailed Student's *t*-test). (C) Cells were treated with various conditioned media alone or with D4476 at 100 μM for 3 h and counted for membrane extensions. Experiments were performed in triplicate with 100 cells counted for each. Columns represent the means and error bars are the S.D.s (***$P<0.001$ using a two-tailed Student's *t*-test). Experiment 4c was performed twice in triplicates. Experiment 4b was performed three times in triplicates.

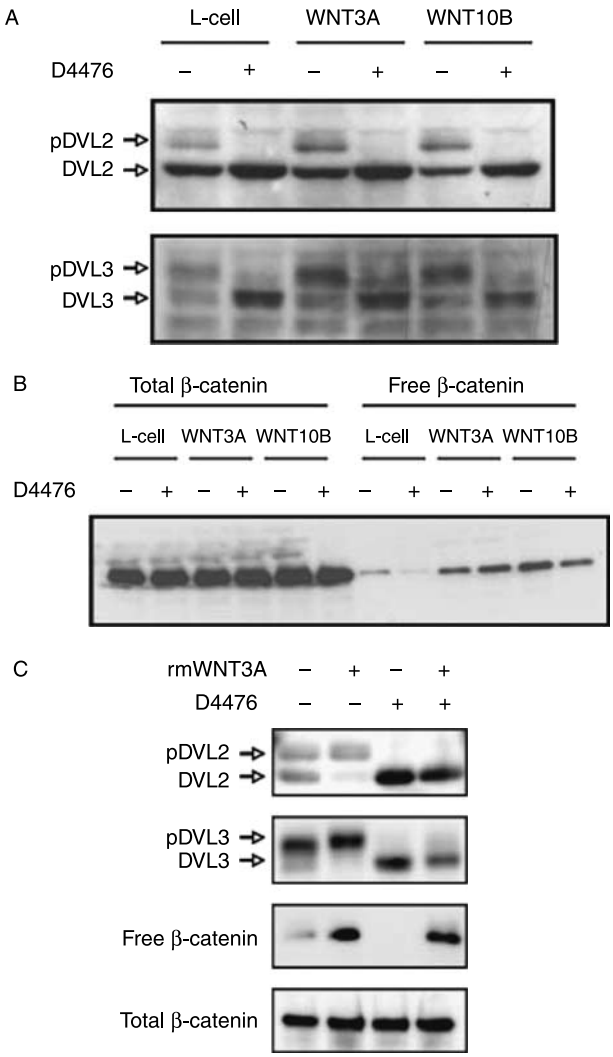

Figure 5 Exogenous Wnts can stimulate Wnt signaling in C18-4 cells as demonstrated by DVL phosphorylation and β-catenin stabilization. CKI inhibitor, D4476, can inhibit DVL phosphorylation but has limited effect on the stabilization of β-catenin. (A) C18-4 cells were treated with L-cell, WNT3A, or WNT10B CM in the presence or absence of D4476 for 3 h. Whole-cell lysates were subject to SDS-PAGE and subsequent immunoblotting with DVL2 and DVL3 antibodies. Unphosphorylated and phosphorylated DVL are denoted by labeled arrows. (B) C18-4 cells were treated with L-cell, WNT3A, or WNT10B CM in the presence or absence of D4476 for 3 h. Free β-catenin was analyzed by GST pull-down experiments using a GST-E-cadherin construct. Experiments were performed in duplicate. (C) C18-4 cells were treated with or without 150 ng/ml of recombinant mouse WNT3A or D4476 in serum-free DMEM for 3 h. Whole-cell lysates were subject to SDS-PAGE and subsequent immunoblotting with DVL2, DVL3, or β-catenin antibodies. Unphosphorylated and phosphorylated DVL are denoted by labeled arrows. Free β-catenin was analyzed by GST pull-down experiments using a GST-E-cadherin construct.

in vitro when other signals were also present (Reya *et al.* 2001, Willert *et al.* 2003). Wnt/β-catenin activation is also observed *in vivo* in self-renewing stem cells as well as in transient-amplifying progenitor cell populations (Alonso & Fuchs 2003, He *et al.* 2004, Tumbar *et al.* 2004). Furthermore, Wnt is also shown to be important for cell differentiation of stem cell precursors (Huelsken *et al.* 2000, Lee & Cho 2002, Otero *et al.* 2004). Therefore, the canonical Wnt pathway does not appear to act on stem cell maintenance that prevents differentiation of stem cells. Recently, a new model has been proposed for Wnt signaling, which better explains the role of Wnt signaling in regulating stem cells (Otero *et al.* 2004, Dravid *et al.* 2005). In this activation model, the canonical Wnt pathway stimulates stem cell activation, survival, or proliferation, which is required for either self-renewal or differentiation. This model for the role of Wnt/β-catenin activation in regulating adult stem cells is further supported by the results in recent papers (Kirstetter *et al.* 2006, Scheller *et al.* 2006, Trowbridge *et al.* 2006).

In the testis, self-renewal or differentiation of SSCs is essential to maintain normal spermatogenesis. However, to date, the mechanisms underlying SSC self-renewal

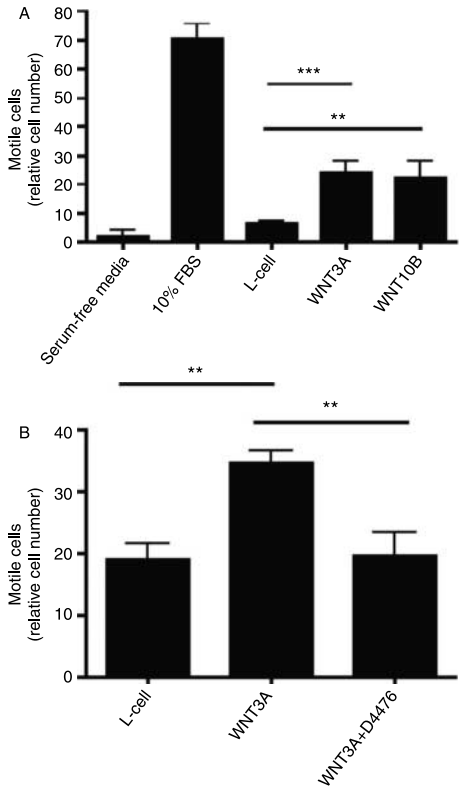

Figure 6 Exogenous WNT3A and WNT10B stimulate chemotaxis in C18-4 cells. Chemotaxis was measured as relative number of cells that had crossed through an 8 μm pore polycarbonate membrane in a Boyden chamber in response to varying media in the lower chamber. Cells that had crossed through the membrane were stained using the Diff-Quik Stain Set (Dade Behring, Deerfield, IL, USA). (A) Increase in motility of C18-4 cells compared to control L-cell CM is statistically significant for WNT3A CM ($P=0.0006$, two-tailed unpaired *t*-test) and WNT10B CM ($P<0.0191$, two tailed unpaired *t*-test). (B) CKI inhibitor, D4476, significantly reduces WNT3A-induced chemotaxis ($P=0.0067$, two-tailed unpaired *t*-test). Experiments were performed twice in triplicate.

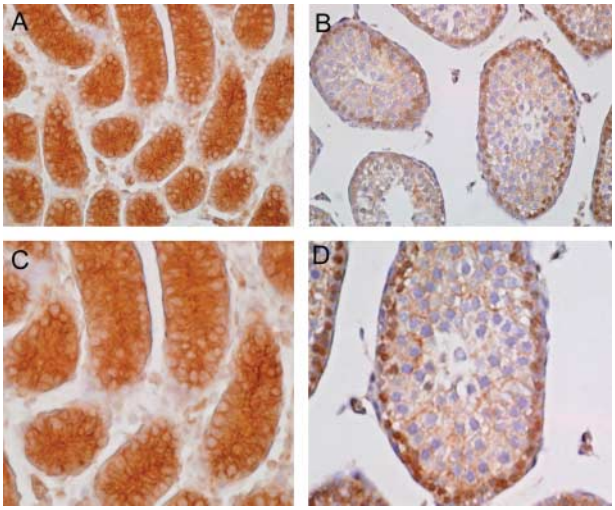

Figure 7 β-catenin is activated during development in mouse spermatogonia. (A) β-catenin staining in the 6-day-old mouse testis, when type A spermatogonia are the only germ cells residing in the seminiferous tubules of the testis. (B) β-catenin staining in 21-day-old mouse testis. Immunohistochemistry with a specific β-catenin antibody showed cytoplasmic and membrane staining for β-catenin in spermatogonia from the 6-day-old testes, whereas 21-day-old testes showed nuclear β-catenin staining. (C) and (D) are higher magnification images of (A) and (B) respectively. Note the unstained nuclei in the spermatogonia from the 6-day-old mouse testis.

versus differentiation remain unclear. Recently, the culture conditions and growth factors essential for self-renewal and expansion of SSCs have been investigated and long-term culture of SSCs has been established (Kanatsu-Shinohara *et al*. 2003, Kubota *et al*. 2004*a*, 2004*b*). It was reported that neonatal mouse SSCs proliferated in culture using a complex, undefined medium containing leukemia inhibitory factor, epidermal growth factor, FGF2, and GDNF. Although these *in vitro* studies suggest the importance of GDNF in self-renewal of SSCs, they do not reflect the complexity of the parameters involved *in vivo* in the testis.

Here, we show that a mouse SSC line (C18-4) expresses *Wnt2*, *Wnt3*, *Wnt3a*, *Wnt7b*, *Wnt8a* as well as *Fzd1*, *Fzd3*, *Fzd4*, and *Fzd5*. We have also demonstrated high expression of *Fzd3* in mouse germ cells, especially in GFRA1-positive cells that were shown to be the SSC (Braydich-Stolle *et al*. 2005). Interestingly, the c-KIT-positive cells that are not considered to be self-renewal SSCs showed a very low expression of *Fzd3*. This observation further confirms the importance of Wnt signaling in the regulation of SSC self-renewal and proliferation. Moreover, WNT3A and WNT10B CM activated the β-catenin pathway in C18-4 cells. It has been reported that the Wnt/β-catenin pathway plays a prominent role in the propagation of hematopoietic stem cells. Among the Wnt proteins demonstrated to have this effect are WNT1, WNT2B, (Willert *et al*. 2003), WNT3A, WNT5A, and WNT10B (Austin *et al*. 1997, Van Den Berg *et al*. 1998, Reya *et al*. 2001, Murdoch *et al*. 2003). Our current study shows that the mouse SSC line (C18-4) is able to respond to WNT3A, WNT10B, and recombinant WNT3A by increasing cell proliferation through the β-catenin pathway. In addition, we showed that WNT3A and WNT10B also increase cell proliferation in primary spermatogonial cell culture.

In most animals, germ line stem cells are established during development following the proliferation and migration of embryonic PGCs (Lin 1997). Cell migration and morphological changes are crucial during normal spermatogenesis. One of the earliest morphological changes during testicular differentiation is the establishment of an XY-specific vasculature. The testis vascular system is derived from mesonephric endothelial cells that migrate into the gonad. In the XX gonad, mesonephric cell migration and testis vascular development are inhibited by WNT4 signaling (Coveney *et al*. 2007). Dishevelled (*Dsh* in *Drosophila* or *Dvl* in mice) is a member of the highly conserved Wnt-signaling pathway. Three orthologous genes of Dishevelled (*Dvl1*, *Dvl2*, and *Dvl3*) have been found in both humans and mice. They play pivotal roles in regulating cell morphology and a variety of changes in cell behaviors. It was shown that the expression of *Dvl1* is stage dependent during mouse spermatogenesis, although *Dvl2* and *Dvl3* show relatively consistent expression (Ma *et al*. 2006). The expression of *Dvl1* mRNA first

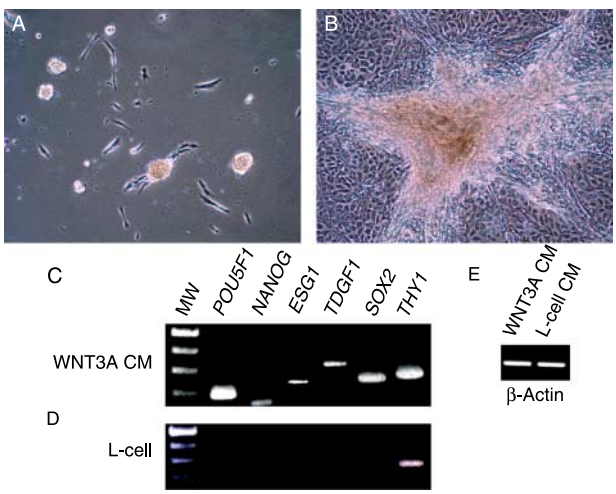

Figure 8 WNT3A-conditioned media maintain human germ cells in an undifferentiated state. (A) Human germ cells cultured for 3 weeks in the presence of WNT3A CM. The ES-like cells were maintained in an undifferentiated state. (B) Human germ cells cultured for 3 weeks in the presence of L-cell CM. The cells were differentiated and stopped forming colonies. (C) RT-PCR analysis of ESC-specific transcription factors in human germ cells cultured for 3 weeks in the presence of WNT3A CM. RT-PCR analysis of ESC-specific markers in human germ cells cultured for 3 weeks in the presence of L-cell CM. (E) RT-PCR analysis of β-actin in human germ cells cultured for 3 weeks in the presence of WNT3A CM or L-cell CM used as internal control. Experiments were performed in triplicate.

appears in pachytene spermatocytes, increases in round and elongating spermatids, and then turns to an undetectable level in mature sperm cells. *DVL1* is present diffusely in the cytoplasm of pachytene spermatocytes and exhibits mainly a vesicular pattern and perinuclear distribution and a weak diffusely cytoplasmic signal in round and elongating spermatids. The vesicular pattern of *DVL1* has been observed in testis somatic cells, and is suggested to play roles in signal transduction. It was also demonstrated that *DVL1* co-immunoprecipitates with β-actin, rather than α-tubulin, in spermatogenic cells suggesting that *DVL1* may be involved in spermatid morphological changes during mouse spermiogenesis through mediating signal transduction and/or regulating actin cytoskeleton organization (Ma *et al*. 2006). In the present study, we have demonstrated that WNT3A CM and WNT10B CM were able to induce morphological changes as well as cell motility in C18-4 cells by triggering the formation of membrane extensions. Interestingly, the morphological changes and cell motility were sensitive to CKI inhibitor, D4476, which completely abolished the formation of membrane extensions and partially but significantly inhibited cell migration. Furthermore, the D4476 was able to completely inhibit the *DVL2* and *DVL3* phosphorylation, yet did not affect the free β-catenin stabilization and accumulation in C18-4 cells. These observations further confirm that the morphological changes and cell motility induced by WNT3A CM and WNT10B CM are owing to the non-canonical Wnt/β-catenin pathway. Figure 9 schematically represents the Wnt pathways that may be involved in SSCs/progenitor cells.

The importance of WNT4 signaling during testicular differentiation was reported. In WNT4 mutant XX gonads, endothelial cells migrate from the mesonephros and form a male-like coelomic vessel. Interestingly, this process occurs in the absence of other obvious features of testis differentiation, suggesting that WNT4 specifically inhibits XY vascular development (Coveney *et al*. 2007). In the present study, we have performed immunostaining of mouse testis with β-catenin antibody at 6- and 21-day-old in order to shed light on the role of Wnt/β-catenin pathway in SSCs during development. The immunohistochemistry revealed membrane staining of β-catenin in spermatogonia of 6-days-old mouse testis, whereas nuclear β-catenin staining was observed in the spermatogonia of 21-day-old mouse testis, confirming the presence of an active β-catenin pathway at this stage. These observations suggest that the β-catenin pathway is activated during development and might regulate the self-renewal and proliferation of SSCs/progenitor cells.

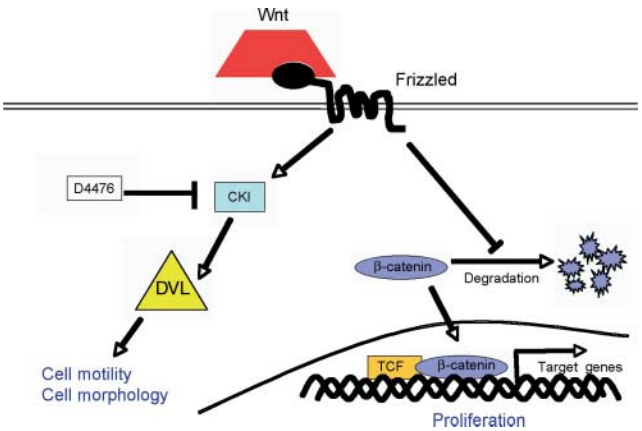

Figure 9 A schematic drawing depicting the Wnt canonical and non-canonical pathways present in spermatogonia.

Although there are studies showing the essential growth factors for mouse and rodent SSCs self-renewal and expansion, to date there is no evidence demonstrating the conservation of SSCs self-renewal signaling between mouse and human. Furthermore, the role of Wnt/β-catenin pathway and especially WNT3A signaling has not yet been examined in the regulation of human SSCs. Here, we demonstrate that human germ cells isolated from adult testis express *WNT3A* as well as *FZD1*, and high expression of *FZD3* and *FZD4* receptor mRNAs. Recent publications have reported the importance of Wnt signaling in maintenance and proliferation of human stem cells. It has been reported that WNT3A can promote human ES cell proliferation in the absence of a feeder layer or conditioned medium (Sato *et al*. 2004). Although WNT3A is important for adult hematopoietic stem cell proliferation (Willert *et al*. 2003, Pinto & Clevers 2005), it is not sufficient to maintain long-term proliferation of human ES cells in the absence of feeder cells (Dravid *et al*. 2005), suggesting a cell-type-dependent role for WNT3A in stem cell maintenance. In addition, a potential role of Wnt/β-catenin signaling in human epidermal stem cells and adult human skin has been implicated suggesting that multipotent stem cells in skin may communicate with their niche partially through Wnt signaling (DasGupta & Fuchs 1999). On the one hand, Wnt appears to be an important factor for stem cell self-renewal and proliferation (Reya *et al*. 2001, Willert *et al*. 2003). On the other hand, Wnt activation is also found to be implicated in stem cell differentiation (Huelsken *et al*. 2000, Lee & Cho 2002, Otero *et al*. 2004). Recently, it has been reported that, in the absence of sufficient levels of factors such as FGF2- or CM-derived factors that are required to sustain an undifferentiated stage, the Wnt activation accelerates cell proliferation as well as differentiation, whereas, in the presence of FGF2 and feeder cells, Wnt stimulates the self-renewing proliferation of the undifferentiated hES cell population (Cai *et al*. 2007). In our study, we have observed similar results for WNT3A inducing cell proliferation and self-renewal of human SSC-derived ES-like cells in the presence of FGF2. Conversely, in the absence of FGF2, WNT3A induced cell differentiation and proliferation. Figure 9 shows the Wnt signaling pathways that may be important in SSCs/progenitor cells.

These observations may be important for the culture of adult human SSCs/progenitor cells and the derived ES-like cells and to sustain the latter in an undifferentiated state. Additional experiments are essential to fully delineate the effect of Wnt/β-catenin and other important signaling pathways in the regulation of adult human SSCs and the ES-like cells derived from these germ cells.

Materials and Methods

The C18-4 cells

The C18-4 cell line was established by transfecting mouse SSCs with a plasmid allowing the expression of the SV40 large T antigen under the control of a ponasterone A-driven promoter (Hofmann *et al*. 2005*a*). It possesses many of the cell surface markers and signal pathways that spermatogonia *in situ* possess (Hofmann *et al*. 2005*b*, Braydich-Stolle *et al*. 2007, He *et al*. 2007, 2008).

Animals

BALB/c mothers with 6- or 14-day-old male pups were obtained from the Charles River Laboratories, Inc (Wilmington, MA, USA). All animal care procedures were carried out pursuant to the National Research Council's Guide for the Care and Use of Laboratory Animals; the experimental protocols were approved by the Animal Care and Use Committee of Georgetown University.

Isolation of type A spermatogonia from 6-day-old mice and germ cells from 60-day-old mice

Type A spermatogonia and Sertoli cells were isolated from the testes of 6-day-old mice using mechanical dissociation and enzymatic digestion with collagenase, trypsin, and hyaluronidase (Bellvé *et al*. 1977, Dym *et al*. 1995). Type A spermatogonia were further separated from the Sertoli cells by differential plating, and the purity of type A spermatogonia was around 95% as evaluated by immunocytochemical staining using an antibody to GCNA1. Adult germ cells were isolated from the testes of 60-day-old mice using a two-step enzymatic digestion containing collagenase, trypsin, and hyaluronidase and differential plating (Bellvé *et al*. 1977, Dym *et al*. 1995).

Isolation of cKit-positive spermatogonia from 14-day-old mice

Germ cells were isolated from 14-day-old mice using the enzymatic digestion described for the 60-day-old mice. c-KIT-positive cells were then purified by MACS using a rabbit polyclonal antibody to cKit (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Typically, the germ cell suspension was resuspended in 1 ml DMEM containing 10% Nu-serum replacement (BD Biosciences, San Jose, CA, USA) and incubated with 25 μl of rabbit anti-c-KIT (C-19) antibody for 16 h at 4 °C with continuous rotation. The cells were washed thrice with ice-cold PBS containing 0.5% BSA and 2 mM EDTA (wash buffer), and then they were resuspended in 80 μl of the wash buffer and incubated with 20 μl of goat anti-rabbit IgG MicroBeads (Miltenyi Biotech, Auburn, CA, USA) for 20 min at 4 °C with rotation. The labeled cells were sorted through a magnetic separation (MS) column attached to a MiniMACS separator (Miltenyi Biotech). c-KIT-positive cells were retained within the column, whereas non-labeled cells passed through. To increase the purity of the separation, the column was washed thrice with 500 μl of wash buffer. In order to collect the c-KIT-positive cells, the column was removed from the magnetic separator and 1 ml of wash buffer was added to the reservoir.

Isolation of germ cells from adult human testis

We obtained human testes from organ donors to the Washington Regional Transplant Consortium. Patients were brain dead with a beating heart and assisted ventilation. The abdomen was opened and the abdominal organs were carefully dissected away from the posterior abdominal wall. The aorta was then clamped and perfused immediately after clamping with Viaspan, a standard organ preservation solution. Viaspan entered all the abdominal organs as well as the testes. The testes and the other organs were retrieved after perfusion for 10 min and packed in cold Viaspan. The testes were sent to the Georgetown University Medical Center by courier. The testes were collected aseptically in serum-free DMEM (high glucose formulation). After decapsulation of the testes, interstitial cells and blood vessels were removed by mechanical agitation and washing after the first enzyme digestion with collagenase IV (Sigma) and DNase I (Sigma) at the concentration of 1 mg/ml and 1 μg/μl respectively. The isolated seminiferous tubules were further digested with collagenase IV, hyaluronidase (Sigma), and trypsin (Sigma) at the concentration of 1, 1.5, and 1 mg/ml respectively to obtain individual germ cells. The germ cells were cultured in ES cell media with DMEM high glucose containing 15% serum replacement, 1× non-essential amino acid, 0.1 mM β-mercaptoethanol (β-ME), 1× penicillin/streptomycin, and 10 ng/ml FGF2. After ∼1 week in culture, small clumps of germ cells adhered to the dish. These possessed many markers of ES-like cells and presumably originated from the SSC/progenitor cell as has been demonstrated in mice (Guan *et al*. 2006, Seandel *et al*. 2007).

Reagents

CKI inhibitor, D4476, was purchased from EMD Chemicals Inc. (San Diego, CA, USA). Unless stated otherwise, all chemical reagents were purchased from Sigma. WNT3A- and WNT10B-CM were collected from L-cells as described by Shibamoto *et al*. (1998), except that serum-free media were used in the present study. The mouse cells were grown on TripleFlasks (Fisher Scientific, Newark, DE, USA) with 500 cm^2 culture area until they were 80% confluent, and then the medium was changed to 100 ml serum-free DMEM. Two successive 72-h harvests of conditioned medium were collected and pooled (Uren *et al*. 2004, Chen *et al*. 2008). Typically, CM was stored at −80 °C; once thawed, medium was kept refrigerated and retained activity for several weeks. Recombinant mouse WNT3A was

purchased from R&D Systems (Minneapolis, MN, USA). DMEM, penicillin/streptomycin, and Serum replacement were purchased from Gibco Invitrogen, FGF2 was purchased from BD Biosciences, and β-ME was purchased from Sigma.

RT-PCR

C18-4 cells, mouse germ cells, and human ES-like cells were collected for total RNA isolation according to the manufacturer's protocol (Promega). Hundred nanograms of DNase-treated RNA were used for first-strand cDNA synthesis. Two microliters of the cDNA reaction were used and amplified for 30 cycles. β-Actin was used as an internal control. Negative controls with RNA containing no cDNA and no RT enzyme were used to perform PCR in order to exclude the possibility of genomic DNA contamination.

Proliferation assay

10^4 C18-4 cells were cultured in 96-well plate in the presence of different CM or recombinant WNT3A for 24–72 h. Proliferation assay was performed using CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega) according to the manufacturer's protocol.

Bromodeoxyuridine (BrdU) incorporation assays

To test the effect of WNT3A and WNT10B on the proliferation of freshly isolated mouse spermatogonia in culture, we isolated spermatogonia from 6-day-old mice and plated them for 16 h on 12-well dishes (~5000 cells/well) in serum-free DMEM-F12 (1:1) at 34 °C with an atmosphere of 5% CO_2. After this starvation period, the following media were added to three different treatment groups; i) L-cell medium, ii) WNT3A CM, and iii) WNT10B CM. Each treatment group consisted of four wells, in three of which we added 20 μg/ml BrdU (Sigma), while the fourth well was used as a 'no BrdU' control. The cells were cultured for 4 days. In the culture, the spermatogonia did not attach. The medium was changed every day by collecting the floating cells, spinning them down, and resuspending them in fresh medium. Finally, the cells were resuspended in Cell Adherence Solution (Crystalgene, Commack, NY, USA) and allowed to attach on glass slides for immunostaining with anti-BrdU. Treatment of the cells for anti-BrdU staining was carried out pursuant to the manufacturer's protocol (Sigma), without the trypsin digest.

Morphological analysis

C18-4 cells were fixed with 3.7% formaldehyde plus 0.1% Triton X-100 for 5 min. Immunofluorescence was performed on C18-4 cells using phalloidin–FITC for actin filament staining. The number of extensions longer than the cell body was counted for each condition and represented in a graph (Uren *et al.* 2004). Human germ cells cultured in the presence and absence of WNT3A CM were analyzed every day and the number of colonies in each well was counted using an inverted microscope.

Immunohistochemistry analysis

Immunohistochemistry with the anti-β-catenin antibody was performed on 5-μm-thick mouse testis sections using the HistoPlus kit (Zymed Laboratories Inc., South San Francisco, CA, USA) according to the manufacturer's protocol.

Chemotaxis assay

The migration assay was performed with the 96-well Boyden chamber MBA96 (Neuro Probe, Inc., Gaithersburg, MD, USA). The bottom wells were filled with 80 μl chemoattractant (L-cell, WNT3A, WNT10B CM, or serum-free media) with or without 100 μM D4476, and an 8 μm porous membrane was placed on top. The top wells were positioned and 200 000 cells in 200 μl serum-free media were added to each well. The chamber was incubated at 37 °C with 5% CO_2 overnight. The chamber was disassembled and the membrane was fixed and stained using the Diff-Quik Stain Set (Dade Behring, Deerfield, IL, USA). The membrane was then washed with water thrice and the cells on the upper surface were gently scraped off. The stained membrane was read directly in the spectrophotometer at 595 nm using a 96-well format.

β-Catenin stabilization

C18-4 cells at 70–80% confluency grown on 10 cm tissue culture dishes were treated with L-cell, WNT3A, or WNT10B CM with or without 100 μM D4476 for 3 h. The cells were then washed with cold PBS, pH 7.4 and lysed in 1 ml lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2.5 mM EDTA, 10 mM NaF, 10 mM $Na_2P_2O_7$, 0.5% NP-40, 1 mM Na_3VO_4, 20 μM aprotinin, 20 μM leupeptin, and 1 mM phenylmethylsulphonyl fluoride). Following a 15 min 12 000 ***g*** centrifugation at 4 °C, the supernatant was transferred into a fresh tube and total protein concentration was determined. Twenty-five micrograms of the lysate were saved as total cell lysate for western blotting. Seven hundred and fifty micrograms of the lysate were precleared by incubating with 40 μl of glutathione-agarose beads for an hour at 4 °C. Samples were centrifuged for 2 min at 12 000 ***g*** at 4 °C and the supernatants were transferred to a fresh tube with 200–300 ng GST-E-cadherin recombinant protein, which contains the cytoplasmic portion of human E-cadherin. Tubes were incubated in a rotator overnight at 4 °C. Then, 40 μl of glutathione-agarose beads were added and incubated for an hour at 4 °C. The beads were washed thrice with 1 ml cold PBS and resuspended in 50 μl 2× sample buffer. Precipitates and 25 μg total lysates were resolved by SDS-PAGE and proteins immunoblotted with 250 ng/ml anti-β-catenin (no. 610154) MAB (BD Biosciences Pharmigen, San Diego, CA, USA).

Dual luciferase assay

β-catenin activation was assessed using the Super8×TOPFlash construct (kindly provided by Dr Randal Moon, University of Washington, Seattle, Washington) and a Renilla-TK mutant construct used as a transfection control (kindly provided by Dr Stephen Byers, Georgetown University). This TOPFlash construct contains eight copies of TCF/LEF-binding sites

upstream of the luciferase gene. An NFKB-responsive construct that has 5×NFKB-binding sites (Stratagene, La Jolla, CA, USA) was used as a negative control. C18-4 cells were transfected using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). A DNA to lipid ratio of 1:2.5 was used. After 24 h of transfection, cells were treated with serum-free DMEM, L-cell, WNT3A-, and WNT10B-CM for 24 h, and then luciferase activity was assessed. All luciferase assays were performed using a dual luciferase assay kit according to the manufacturer's protocol (Promega).

Disheveled phosphorylation

From the β-catenin stabilization assay, 50–100 μg of total lysate were resolved by SDS-PAGE and proteins immunoblotted with 200 ng/ml of anti-DVL2 (H75) and anti-DVL3 (4D3) antibodies (Santa Cruz Biotechnology). DVL phosphorylation was observed as a band shift (Gonzalez-Sancho *et al*. 2004).

Declaration of interest

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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