AIP1-mediated actin disassembly is required for postnatal germ cell migration and spermatogonial stem cell niche establishment

J Xu1,4,5, P Wan1,5,6, M Wang2,5, J Zhang1, X Gao1, B Hu1, J Han1, L Chen1, K Sun3, J Wu*3, X Wu*2, X Huang*1,7 and J Chen*1

In mammals, spermatogonial stem cells (SSCs) arise from early germ cells called gonocytes, which are derived from primordial germ cells during embryogenesis and remain quiescent until birth. After birth, these germ cells migrate from the center of testicular cord, through Sertoli cells, and toward the basement membrane to form the SSC pool and establish the SSC niche architecture. However, molecular mechanisms underlying germ cell migration and niche establishment are largely unknown. Here, we show that the actin disassembly factor actin interacting protein 1 (AIP1) is required in both germ cells and Sertoli cells to regulate this process. Germ cell-specific or Sertoli cell-specific deletion of Aip1 gene each led to significant defects in germ cell migration after postnatal day 4 or 5, accompanied by elevated levels of actin filaments (F-actin) in the affected cells. Furthermore, our data demonstrated that interaction between germ cells and Sertoli cells, likely through E-cadherin-mediated cell adhesion, is critical for germ cells’ migration toward the basement membrane. At last, Aip1 deletion in Sertoli cells decreased SSC self-renewal, increased spermatogonial differentiation, but did not affect the expression and secretion levels of growth factors, suggesting that the disruption of SSC function results from architectural changes in the postnatal niche.

Cell Death and Disease (2015) 6, e1818; doi:10.1038/cddis.2015.182; published online 16 July 2015

In mammals, spermatogenesis and male fertility depend on the self-renewing and differentiating functions of spermatogonial stem cells (SSCs), which are regulated by cues from the niche microenvironment.1 During embryogenesis, the precursors of SSCs can be traced to primordial germ cells (PGCs) in the proximal epiblast at embryonic day 6.25 (E6.25), which migrate to genital ridge and together with somatic cells there to form the embryonic gonad.2 The PGCs then differentiate to gonocytes (also called prespermatogonia), proliferate for a brief period of time, and then remain mitotically quiescent until birth.3–5 After birth, these neonatal germ cells (gonocytes) located at the center of testicular cord become proliferative and relocate themselves from the center toward the basement membrane of each testicular cord.4,6 During the migration or relocation process, germ cells associate with and move through the Sertoli cells, the sole somatic cell type within the testicular cord and the major component of the SSC niche. After reaching the basement membrane at the periphery, most of these germ cells adopt a distinct morphology and become the undifferentiated spermatogonial population, which includes SSCs and other non-stem cell progenitors,7–9 supposedly in response to cues from the supporting cells. It has been suggested that the postnatal germ cell migration is crucial for the formation of SSC pool and the establishment of the SSC niche architecture. However, the mechanisms underlying these two processes are not well understood.

In neonatal mice, germ cells specifically express the cell adhesion molecule E-cadherin on the cell surface,10,11 whereas other adhesion markers including N-cadherin and β1-integrin were found in both germ cells and Sertoli cells.12–14 However, whether these adhesion molecules have specific roles in germ cells’ outward migration and subsequent differentiation were not yet known. In Drosophila testis, the germ line stem cells (GSCs) were shown to attach to the somatic hub cells (a major niche component) via membrane bound E-cadherin in both cell groups, and disruption of E-cadherin-mediated cell adhesion between GSCs and hub cells severely affected self-renewal and maintenance of GSCs.15,16 Moreover, a recent study showed that the actin polymerization regulator profilin is required to localize and maintain E-cadherin to the GSC-hub cell interface and is thus essential for the maintenance of GSCs. This result

1 State Key Laboratory of Pharmaceutical Biotechnology and MOE Key Laboratory of Model Animals for Disease Study, Model Animal Research Center, Nanjing University, Nanjing, China; 2 State Key Laboratory of Reproductive Medicine, Nanjing Medical University, Nanjing, China; 3 Bio-X Institute, Shanghai Jiaotong University, Shanghai, China
4 Corresponding author: J Chen or X Huang or X Wu or J Wu, State Key Laboratory of Pharmaceutical Biotechnology and MOE Key Laboratory of Model Animals for Disease Study, Model Animal Research Center, Nanjing University, 12 Xue-fu Road, Nanjing 210061, China. Tel/Fax: +86 25 58641507; E-mail: chenjiong@nju.edu.cn or huangxx@shanghaitech.edu.cn or xinwu@njmu.edu.cn or jiwu@sjtu.edu.cn
5 Present address: Nanjing Maternity and Child Health Care Hospital, Nanjing Medical University, Nanjing, China.
6 Present address: School of Life science, Jiangxi Science & Technology Normal University, Nanchang, China.
7 Present address: School of Life Science and Technology, ShanghaiTech University, Shanghai, China.
8 These authors contributed equally to this work.
9 Abbreviations: SSCs, spermatogonial stem cells; AIP1, actin-interacting protein 1; F-actin, actin filaments; PGCs, primordial germ cells; GSCs, germline stem cells; ADFs, actin-depolymerizing factors; HE, hematoxylin and eosin; MACS, magnetic-activated cell sorting; BTB, blood-testis barrier

Received 23.10.14; revised 18.5.15; accepted 19.5.15; Edited by M Agostini
is consistent with findings in other systems that dynamics of actin cytoskeleton directly regulate the assembly and maintenance of E-cadherin-based cell adhesion. Interestingly, we have previously shown that actin interacting protein 1 (AIP1), an actin disassembly factor, regulates E-cadherin distribution and dynamics during a cell rearrangement process of the Drosophila eye disc. AIP1 has been shown to act together with cofilin/actin-depolymerizing factors to promote actin dynamics in various cellular processes, and it is highly conserved in all eukaryotes examined so far. Here, we utilized germ cell- or Sertoli cell-specific deletion of Aip1 (also known as Wdr1) in the murine testis to study the process of germ cell migration and SSC niche establishment.

Results

Aip1 deletion in Sertoli cells or germ cells caused severe defects in spermatogenesis. First, we utilized the Aip1 (Wdr1) conditional knockout mouse model (Aip1lox/lox), which contained loxP sites flanking the exon 4 of the gene, leading to the premature termination of Aip1 translation (details of the conditional knockout construct has been reported by Yuan et al.25). To achieve deletion of Aip1 specifically in early developing testis, we crossed the Aip1lox/lox with Amhl (anti-müllerian hormone)-cre mice that express the cre recombinase in Sertoli cells starting from embryonic day 15 (E15). To obtain germ cell-specific Aip1 knockout, we crossed Aip1lox/lox with Vasa-cre mice that express cre in the germline beginning from E15. Western blot analysis (with a previously referenced anti-AIP1 antibody) of THY1+ germ cells extracted from testes of postnatal day 7 (P7) Aip1lox/lox: Vasa-cre (referred to as geAip1−/− subsequently) mice showed an almost complete removal of the AIP1 protein, indicating an efficient deletion of Aip1 in the germ cells (Figure 1a). Western blot of Sertoli cells from testes of P7 Aip1lox/lox:Amhl-cre (seAip1−/−) mice exhibited 60% reduction of AIP1 protein level, indicating a partial loss-of-function of Aip1 in the Sertoli cells (Figure 1b). Finally, the marked increase of F-actin levels, as shown by phalloidin staining, in the P7 geAip1−/− germ cells (Figure 1c) and P7 seAip1−/− Sertoli cells (Figure 1d), are highly consistent with our previous characterization of Aip1’s loss-of-function actin phenotypes in Drosophila and mouse.18,25

Hematoxylin and eosin (HE) staining revealed that the earliest observable defects occurred at P7 when a significant portion of germ cells in both geAip1−/− and seAip1−/− testes failed to relocate to the basement membrane (Figure 2a). Further HE and immunofluorescence analyses from P12 to P25 showed that Aip1 ablation in the germ cells and Sertoli cells both caused severe defects in later stages of spermatogenesis and Aip1 was likely required for the later proliferation and meiosis of germ cells (Figure 2 and Supplementary Figure 1).

Aip1 is required in both Sertoli cells and germ cells for the outward migration of germ cells to basement membrane. We next focused our analysis on the outward migration of germ cells between P2 and P7 for both geAip1−/− and seAip1−/− testes, as deficit in this process is the earliest phenotype we detected and the subsequent severe spermatogenesis phenotypes could be the secondary consequence of earlier defects. To analyze the positioning of germ cells and Sertoli cells within the testicular cord, we immunostained sectioned testis tissues for PLZF (undifferentiated spermatagonia marker29,30), mouse vasa homolog (MVH, general germ cell marker26) and GATA4 (Sertoli cell and Leydig cell marker31). In P2 control testis, most of round germ cells (gonocytes) labeled with MVH were shown to localize within the center of testicular cord, whereas Sertoli cells were positioned close to the basement membrane. At P4, a major portion of PLZF-labeled germ cells could be seen to relocate to the basement membrane. By P7, most of the germ cells had finished the outward migration, resulting in the large round spermatagonia intermingling with the smaller and irregularly shaped Sertoli cells adjacent to the basement membrane (Figure 3a). To quantify this process of germ cell migration, we counted both the number of germ cells that have reached the basal region of testicular cord (Nbasal) and the number of germ cells that have not reached the basement membrane and thus remain in the central region (Ncentral) for each cross-sectional view of one testicular cord. In the control testes, we found that the total number of germ cells (Ncentral+Nbasal) per section increased from 2.21 ± 0.19 (n = 51) at P2 to 7.67 ± 0.38 at P7 (n = 52) (Figure 3b). Such a 3.5-fold increase of germ cell number indicates that postnatal germ cells proliferate during their migration, which
is consistent with previous findings. In the mutant testes, the total number of germ cells increased in a similar fashion as the controls, from $2.28 \pm 0.21$ at P2 to $7.15 \pm 0.41$ at P7 (for geAip1$^{-/-}$) and from $3.29 \pm 0.27$ at P2 to $7.24 \pm 0.44$ at P7 (for seAip1$^{-/-}$), respectively (Figure 3b and c), suggesting that Aip1 deletion in either germ cells or Sertoli cells resulted in no major proliferation defects. Indeed, double staining with Ki67 (proliferative marker) and PLZF for the control and the mutant testes confirmed that no significant proliferation defect occurred in the germ cells in either geAip1$^{-/-}$ or seAip1$^{-/-}$ testes (Supplementary Figures 2 and 3). However, the $N_{basal}$ value, which reflects the extent of germ cell migration, was decreased after P4 or P5 for geAip1$^{-/-}$ or seAip1$^{-/-}$ testes, respectively. From P2 to P4, there were no significant differences in the $N_{basal}$ or $N_{central}$ values between the mutant (geAip1$^{-/-}$ or seAip1$^{-/-}$) and control testes, indicating that germ cells in both mutants migrate normally from P2 to P4. But beginning at P5, $N_{basal}$ of geAip1$^{-/-}$ testes was significantly decreased as compared with the control, and the decrease became most severe at P7. On the other hand, $N_{central}$ value began to display significant increase at P5 for geAip1$^{-/-}$ testes (Figure 3b). Similarly, reduction in $N_{basal}$...
value and increase in $N_{\text{central}}$ also took place for $seAip1^{-/-}$ testes beginning at P6 and P5, respectively (Figure 3c). Taken together, these results demonstrate that $Aip1$ is autonomously required in the germ cells for their migration after P4. Interestingly, $Aip1$ is also non-autonomously required in the supporting Sertoli cells for germ cell migration. Furthermore, germ cells' migration defects in both $geAip1^{-/-}$ and $seAip1^{-/-}$ testes were not due to the effects of $Aip1$ ablation on cell proliferation of either germ cells or Sertoli cells.

We next determined what aspects of germ cell migration were prominently affected by $Aip1$ deletion in either germ cells or Sertoli cells. Consistent with AIP1's roles in promoting actin dynamics in various model organisms, we found that $Aip1$ deletion caused actin disassembly defects and ectopic F-actin patches in Sertoli cells and germ cells within testes of $seAip1^{-/-}$ and $geAip1^{-/-}$ mice, respectively (Figure 1c and d). The ectopic F-actin phenotype in both $seAip1^{-/-}$ and $geAip1^{-/-}$ testes began at P4 and became more severe in older mice (Supplementary Figure 4), which is consistent with the increasingly severe migration defects in the mutant testes. Immunostaining with antibody against β-actin also verified that the abnormal actin patches were enriched in the mutant testes (Figure 4a and b). In the P5 control testis, β-actin staining revealed that a significant portion of germ cells (15 out of 59 in P5 control) that have yet to reach the basement membrane

Figure 3  AIP1 is required in both germ cells and Sertoli cells for germ cells migration during postnatal testis development. (a) Germ cells from $geAip1^{-/-}$ and $seAip1^{-/-}$ testes displayed significant defects in their migration as compared with their respective control. Control germ cells (labeled by MVH staining, green) at P2 mostly remained in the central of each tubule, whereas migratory germ cells (labeled by PLZF, green) were observed at P4–P5. By P6 or P7, most of the germ cells had reached and contacted the basement membrane of the testicular cord. In comparison, germ cells in the $geAip1^{-/-}$ and $seAip1^{-/-}$ testes showed similar localization at P2 and P4 as that of the controls, but displayed significant migration delay starting from P5 or P6. GATA4 staining labels the Sertoli cells (red). White arrows point to examples of germ cells that had not reached the basement membrane. Scale bar: 10 μm. (b and c) Number of germ cells that were located at either the basal or central region of the testicular cord was quantified in $geAip1^{-/-}$ (b) and $seAip1^{-/-}$ (c) testes from P2 to P7 with respect to their controls. Each image was taken from a 5-μm-thick tissue section. A total of 51 tissue cross-sections were analyzed for each genotype at a certain time point (from three mice, except for P2 $geAip1^{-/-}$). Data are presented as means ± S.E.M., *$P<0.05$, **$P<0.01$, ***$P<0.001$
extended polarized and actin-rich lamellipodial protrusions toward the basement membrane (between Sertoli cells or between Sertoli cells and germ cells) during their migration (Figure 4a and B). In P5 geAip1−/− testes, these polarized lamellipodial protrusions with membrane-enriched actin staining were much less likely to be observed (15 out of 183). Instead, random (not polarized toward the basal region) protrusions and cell cortical regions filled with intensely stained actin patches were more frequently (62 out of 183) observed in germ cells that have not reached the basement membrane (Figure 4a). At last, in Sertoli cells of seAip1−/− testes, cell cortical regions were often observed to be filled with large ectopic patches of intense actin staining beginning at P4 (Figure 4b, Supplementary Figure 4). Together, these data indicate that actin disassembly mediated by AIP1 is required in the cell peripheral and lamellipodial regions of germ cells and Sertoli cells in the wild-type testis to promote outward migration of germ cells.

**Aip1 deletion caused specific E-cadherin distribution defects in germ cells and Sertoli cells.** As cell–cell and cell–substrate adhesions are critical for cell migration, we then examined the distribution patterns of cell adhesion markers in both control and mutant cells. We found that E-cadherin was strongly expressed in PLZF-labeled spermatogonia with a continuous and uniform E-cadherin staining on the cell membrane. In geAip1−/− testes, a significant portion (21%, n = 15 sections from three mice) of P7 germ cells displayed uneven and discontinuous membrane staining on the cell surface. Moreover, ectopic patches of strong E-cadherin staining were found near the cell surface (Figure 5a and c). Intriguingly, such defects of E-cadherin distribution in the germ cells were similarly found in seAip1−/− testes (Figure 5b), indicating that AIP1 loss-of-function in Sertoli cells had a non-autonomous effect on germ cells. In addition, we found ectopic E-cadherin staining on the membrane of a significant portion (13.3%, n = 23) of P7 mutant Sertoli cells (indicated by SOX9, a Sertoli cell marker[32], whereas control Sertoli cells expressed only very little detectable E-cadherin staining (only 2%, n = 18) (Figure 5d). Culturing of control and seAip1−/− Sertoli cells also confirmed the ectopic increase of E-cadherin level in the mutant Sertoli cells (Supplementary Figure 5). Surprisingly, significant upregulation of E-cadherin was also detected in Sertoli cells in the P7 geAip1−/− testes (Figure 5c and Supplementary Figure 6), indicating that Aip1-deleted germ cells had a non-autonomous effect on the wild-type Sertoli cells. Next, we found that N-cadherin and β1-integrin, two prominent adhesive markers that are uniformly localized on the cell surface of both germ cells and Sertoli cells, distributed normally for both types of cells in geAip1−/− and seAip1−/− testes (Figure 6a–d). Taken together, these data suggested that the cytoskeletal changes due to AIP1 deficiency caused specific defects in E-cadherin localization for both germ cells and Sertoli cells and that E-cadherin or cytoskeletal defects in one cell type non-autonomously affected E-cadherin distribution of the other cell type.

**Aip1 deletion in Sertoli cells affected SSC self-renewal and spermatogonial differentiation.** The stem cell niche is essential for stem cell functions. To determine the effects of Sertoli cells on self-renewal of SSCs and differentiation of spermatogonia, we examined a variety of stem cell and cell differentiation markers in seAip1−/− testes at P4, P7, P9 and P12. We first identified SSCs among undifferentiated spermatogonia in both control and seAip1−/− testes by double labeling of PLZF and OCT4 (a marker of SSC in testes[33,34]). Consistent with previous reports, OCT4 was only expressed in a subset of PLZF-positive cells, and all OCT4-positive cells are also PLZF-positive. Thus, we considered the OCT4 and PLZF double-positive cells as the SSCs. The number of SSCs in seAip1−/− testes was not significantly different from control testes at P4 and P7. However, the number of OCT4 and PLZF double-positive cells as well as...
the PLZF-positive cells reduced significantly in P9 seAip1−/− testes when compared with the control (Figure 7a, b, d and e, Supplementary Figure 7). However, the number of all germ cells that were labeled by MVH (general germ cell marker36) remained unchanged in seAip1−/− testes at P7, P9 and P12 (Figure 7b and f), suggesting that the reduction of SSCs was

![Image of Figure 5](image-url)

**Figure 5**  Ap1 deletion in either germ cells or Sertoli cells caused alterations in E-cadherin distribution pattern in both germ cells and Sertoli cells. (a and b) Co-immunostaining of E-cadherin (red) and PLZF (green) of testis sections from the P7 control, geAip1−/− (a) and seAip1−/− (b) mice. (c and d) Co-immunostaining of E-cadherin (red) and SOX9 (Sertoli cell marker, green) of testis sections from the P7 control, geAip1−/− (c) and seAip1−/− (d) mice. White arrows point to regions of Sertoli cells where E-cadherin level is upregulated, and the asterisk (*) signs mark germ cells whose E-cadherin distribution pattern is affected. Cell nuclei were stained with DAPI (blue). Scale bars: 10 μm
specific and was not a secondary consequence of reduction in the general germ cell population. At last, the marked reduction of SSC number at P9 cannot be explained by apoptosis, as there was no significant increase of activated Caspase 3 and PLZF double-positive cells in the P9 or P12 seAip1−/− testes (Figure 7c and g). Together, these data indicated that SSCs’ self-renewing ability was non-autonomously affected by mutant Sertoli cells.

We next characterized the differentiation profile by real time (RT)-PCR analysis of the expression of c-kit,37–39 the marker for differentiating spermatogonia, and Stra8,40 the marker for pre-meiotic germ cells in the whole testes. We found that the expression of both c-kit and Stra8 increased significantly in seAip1−/− testes at P9 (Figure 7h and i). Furthermore, flow cytometry data showed that the percentages of c-Kit+ and PLZF+ cells among the whole testes were significantly increased and decreased, respectively (Figure 7j and k, Supplementary Figure 7), confirming an increase of spermatogonial differentiation and decrease of spermatogonial self-renewal ability in the mutant testes. It was commonly understood that growth factors and chemokines secreted by Sertoli cells and other types of cells in the niche directly regulate the self-renewal and differentiation of SSCs. We next examined the expression levels of a variety of growth factors and chemokines that had been previously shown to affect SSC function. RT-PCR analysis showed that expression levels of Gdnf,41 Cxcl12,42–44 Fgf2,45,46 Egf47 and Scf48,49 displayed no significant difference between seAip1−/− and control testes (Supplementary Figure 8A). Furthermore, ELISA assay detected no significant alteration in the protein levels of secreted GDNF and FGF2 by isolated Sertoli cells from the P7 seAip1−/− testes (Supplementary Figure 8B). At last, transcript levels of Fshr, Lhcg, Esr1 and Ar, which encode receptors for hormones FSH, LH, estrogen and androgen, respectively,50,51 were not significantly changed in seAip1−/− testes except for only mild reduction in levels of Fshr at P8 and P9, Ar at P9 (Supplementary Figure 8C). These data suggested that the loss-of-function of AIP1 and the resulting SSC niche architecture change did not significantly alter the expression or secretion of growth factors or hormonal...
Figure 7  Aip1 deletion in Sertoli cells resulted in increase of spermatogonial differentiation and decrease of SSC self-renewal. (a) Co-immunostaining of OCT4 and PLZF in P4, P7 and P9 seAip1+/− and control testes. The result was quantified in (d). (b) Co-immunostaining of MVH and PLZF in P7, P9 and P12 seAip1+/− and control testes, with results quantified in (e) and (f). (c) Co-immunostaining of activated caspase-3 and PLZF in P7, P9 and P12 seAip1+/− and control testes, with results quantified in (g). (d–g) Quantification of SSC (d; PLZF and OCT double positive), undifferentiated spermatogonia (e; labeled by PLZF), germ cells (f; labeled by MVH) and apoptotic spermatogonia (g; PLZF and activated caspase-3 double positive) per cross-section of each testicular cord for both seAip1+/− and control mice at indicated postnatal dates. A total of 51 tissue cross-sections were analyzed for each genotype at a certain time point (from three mice, except for P12 in c and g). (h) and (i) Relative expression levels of c-Kit (h) and Stra8 (i) from RT-PCR analysis. (j) Increased differentiation as demonstrated by flow cytometry analysis of the c-Kit+ cells. (k) Percentage of c-Kit+ cells from flow cytometry data. (l) Scale bars: 50 μm. Data are presented as means ± S.E.M.; *P < 0.05, **P < 0.01, ***P < 0.001.
The recipient testis that was transplanted with cells from seminiferous tubules from recipient testes (Figures 8b and c). Surprisingly, germ cells from mutant mice germ cell clumps from testes of their normal stem cell function. To determine whether the effects on the SSCs, we performed germ cell transplantation, F1 mice were used as germ cell transplant recipients. Figures 9A and B). The busulfan-treated C57BL/6 × 129sv P7 sorting (MACS) to enrich SSCs 54,55 (Supplementary Figure 9). Taken together, these data demonstrated that defects in the niche microenvironment caused by Aip1 deletion in Sertoli cells did not produce irreversible damage to the SSC function.

**Discussion**

SSC’s migration, in the context of homing to the germline niche, has recently been extensively studied. This migration event requires RAC1 and β1-integrin, but not E-cadherin in the spermatogonia, and it also requires β1-integrin in the Sertoli cells. However, during homing in the adult testis, SSCs need to cross the blood-testis barrier (BTB), which is composed of tight junctions between Sertoli cells. The migration of gonocytes occurs during the postnatal period and before the inter-Sertoli cells tight junction-BTB establishment (at P12-P14). At present, the underlying mechanism for postnatal germ cell migration is mostly unknown and it is supposed to be different from that of SSC homing. Here, we found that postnatal germ cell migration required AIP1 in both germ cells and Sertoli cells. Ectopic actin accumulations were apparent for both cell types beginning at P4. The onset of the actin defects correlated well with the onset of the migration defects. Our data indicated that impairment of actin disassembly could directly affect postnatal germ cell migration in two major ways.

First, ectopic actin patches were often found in the lamellipodia-like protrusions of migrating Aip1−/− germ cells, indicating the functional importance of the actin-based protrusions. A previous study reported that neonatal rat gonocytes with pseudopod extension were identified in the rat testis cell suspensions in vitro and these cells could colonize niche of recipient testis much more efficiently than gonocytes without pseudopod. But similar pseudopod extensions in gonocytes had not been detected in mouse. Our study provided important in vivo evidence that actin-rich protrusions existed in the postnatal germ cells and that these protrusions probably required robust actin turnover to drive germ cells’ migration toward the basement membrane.

Second, the lack of actin dynamics could disrupt E-cadherin-mediated cell adhesion, leading to block of germ cells migration. Our data showed that the distribution pattern of E-cadherin but not N-cadherin and β1-integrin was significantly affected in Aip1−/− germ cells and Sertoli cells. It is known that the membrane distribution and clustering of E-cadherin molecules are regulated by the dynamics of the F-actin microfilament assembly. It was found that postnatal germ cell migration required AIP1 in both germ cells and Sertoli cells. Ectopic actin accumulations were apparent for both cell types beginning at P4. The onset of the actin defects correlated well with the onset of the migration defects. Our data indicated that impairment of actin disassembly could directly affect postnatal germ cell migration in two major ways.

First, ectopic actin patches were often found in the lamellipodia-like protrusions of migrating Aip1−/− germ cells, indicating the functional importance of the actin-based protrusions. A previous study reported that neonatal rat gonocytes with pseudopod extension were identified in the rat testis cell suspensions in vitro and these cells could colonize niche of recipient testis much more efficiently than gonocytes without pseudopod. But similar pseudopod extensions in gonocytes had not been detected in mouse. Our study provided important in vivo evidence that actin-rich protrusions existed in the postnatal germ cells and that these protrusions probably required robust actin turnover to drive germ cells’ migration toward the basement membrane.

Second, the lack of actin dynamics could disrupt E-cadherin-mediated cell adhesion, leading to block of germ cells migration. Our data showed that the distribution pattern of E-cadherin but not N-cadherin and β1-integrin was significantly affected in Aip1−/− germ cells and Sertoli cells. It is known that the membrane distribution and clustering of E-cadherin molecules are regulated by the dynamics of the F-actin microfilament assembly. It was found that postnatal germ cell migration required AIP1 in both germ cells and Sertoli cells. Ectopic actin accumulations were apparent for both cell types beginning at P4. The onset of the actin defects correlated well with the onset of the migration defects. Our data indicated that impairment of actin disassembly could directly affect postnatal germ cell migration in two major ways.

Transplantation restored SSCs from seAip1−/− testes to their normal stem cell function. To determine whether the disrupted niche in seAip1−/− testes could result in irreversible effects on the SSCs, we performed germ cell transplantation, which was considered as effective means to examine the SSC function. THY1+ germ cells were isolated from P7–P8 testes of seAip1−/−:Rosa26-mT/mG and Aip1+/−:Rosa26-mT/mG donor mice by magnetic-activated cell sorting (MACS) to enrich SSCs (Supplementary Figures 9A and B). The bsulfan-treated C57BL/6 J × 129sv F1 mice were used as germ cell transplant recipients. Two months after transplantation with sorted THY1+ cells, the germ cell colonies with red fluorescence (from mT) were found in the control testes and counted (Figure 8b and c). Surprisingly, germ cells from mutant mice produced 1.5–3.2 folds more colonies in the recipient testes than the control (Table 1). HE staining of the recipient testes showed that there were germ cells of different stages (Figure 8d), indicating that normal spermatogenesis of donor germ cells took place. Furthermore, in vitro proliferation assay showed that THY1+ germ cells from mutant testes retained the SSC potential to form colonies and had comparable growth rates as control germ cells (Figure 8a, Supplementary Figure 9). Taken together, these data demonstrated that defects in the niche microenvironment caused by Aip1 deletion in Sertoli cells did not produce irreversible damage to the SSC function.

**Discussion**

SSC’s migration, in the context of homing to the germline niche, has recently been extensively studied. This migration event requires RAC1 and β1-integrin, but not E-cadherin in the spermatogonia, and it also requires β1-integrin in the Sertoli cells. However, during homing in the adult testis, SSCs need to cross the blood-testis barrier (BTB), which is composed of tight junctions between Sertoli cells. The migration of gonocytes occurs during the postnatal period and before the inter-Sertoli cells tight junction-BTB establishment (at P12-P14). At present, the underlying mechanism for postnatal germ cell migration is mostly unknown and it is supposed to be different from that of SSC homing. Here, we found that postnatal germ cell migration required AIP1 in both germ cells and Sertoli cells. Ectopic actin accumulations were apparent for both cell types beginning at P4. The onset of the actin defects correlated well with the onset of the migration defects. Our data indicated that impairment of actin disassembly could directly affect postnatal germ cell migration in two major ways.

First, ectopic actin patches were often found in the lamellipodia-like protrusions of migrating Aip1−/− germ cells, indicating the functional importance of the actin-based protrusions. A previous study reported that neonatal rat gonocytes with pseudopod extension were identified in the rat testis cell suspensions in vitro and these cells could colonize niche of recipient testis much more efficiently than gonocytes without pseudopod. But similar pseudopod extensions in gonocytes had not been detected in mouse. Our study provided important in vivo evidence that actin-rich protrusions existed in the postnatal germ cells and that these protrusions probably required robust actin turnover to drive germ cells’ migration toward the basement membrane.

Second, the lack of actin dynamics could disrupt E-cadherin-mediated cell adhesion, leading to block of germ cells migration. Our data showed that the distribution pattern of E-cadherin but not N-cadherin and β1-integrin was significantly affected in Aip1−/− germ cells and Sertoli cells. It is known that the membrane distribution and clustering of E-cadherin molecules are regulated by the dynamics of the F-actin microfilament assembly. It was found that postnatal germ cell migration required AIP1 in both germ cells and Sertoli cells. Ectopic actin accumulations were apparent for both cell types beginning at P4. The onset of the actin defects correlated well with the onset of the migration defects. Our data indicated that impairment of actin disassembly could directly affect postnatal germ cell migration in two major ways.

Transplantation restored SSCs from seAip1−/− testes to their normal stem cell function. To determine whether the disrupted niche in seAip1−/− testes could result in irreversible effects on the SSCs, we performed germ cell transplantation, which was considered as effective means to examine the SSC function. THY1+ germ cells were isolated from P7–P8 testes of seAip1−/−:Rosa26-mT/mG and Aip1+/−:Rosa26-mT/mG donor mice by magnetic-activated cell sorting (MACS) to enrich SSCs (Supplementary Figures 9A and B). The bsulfan-treated C57BL/6 J × 129sv F1 mice were used as germ cell transplant recipients.

Two months after transplantation with sorted THY1+ cells, the germ cell colonies with red fluorescence (from mT) were derived from the donor testes were examined and counted (Figure 8b and c). Surprisingly, germ cells from mutant mice produced 1.5–3.2 folds more colonies in the recipient testes than the control (Table 1). HE staining of the recipient testes showed that there were germ cells of different stages (Figure 8d), indicating that normal spermatogenesis of donor germ cells took place. Furthermore, in vitro proliferation assay showed that THY1+ germ cells from mutant testes retained the SSC potential to form colonies and had comparable growth rates as control germ cells (Figure 8a, Supplementary Figure 9). Taken together, these data demonstrated that defects in the niche microenvironment caused by Aip1 deletion in Sertoli cells did not produce irreversible damage to the SSC function.
removing the function of profilin, an actin polymerization-promoting factor, also caused disruption of E-cadherin staining in the interface between GSCs and the supporting hub cells.17

In agreement with the above reports, our data indicated that Aip1 deletion resulted in the disruption of E-cadherin distribution in the interface between germ cells and Sertoli cells, which could lead to their migration defects. Interestingly, Aip1 deletion in Sertoli cells also led to ectopic patches of E-cadherin staining near the cell membrane of Sertoli cells as well as discontinuous E-cadherin staining (but without ectopic patches) in the cell membrane of the spermatogonia. The autonomous effect of strong increase of membrane-associated or ectopically located E-cadherin could be explained if robust actin dynamics is required to promote fast turnover of membrane-associated E-cadherin in the wild-type Sertoli cells (probably via endocytosis). Indeed, actin dynamics has been shown to be critical for endocytosis.59

Furthermore, the non-autonomous effect on spermatogonia’s E-cadherin distribution could be due to the altered pattern of E-cadherin localization in the adjacent Sertoli cells and the homophilic nature of the trans-membrane E-cadherin.

Our data from Aip1 deletion in the Sertoli cells also provided important insights into how the SSC niche in the neonatal testis is established and how the niche influences SSC self-renewal and differentiation. The niche is commonly thought of consisting of two parts, namely a growth factor milieu and architectural support. The Sertoli cell-specific ablation of Aip1 did not significantly alter the expression and secretion levels of growth factors in the Sertoli cells. However, germ cell migration toward the basement membrane was impaired, beginning at P5. This result suggested that disruption of germ cells migration results in defects in the establishment of the initial (neonatal) SSC niche, eventually leading to impairment of SSC function. This is possibly caused by the changes in niche architecture, which were manifested as alterations in the actin distribution in Sertoli cells and E-cadherin distribution in both the supporting Sertoli cells and spermatogonia. Interestingly, the architectural and adhesive alterations in the niche did not result in a permanent impairment of SSC function, as transplanting the SSCs taken from seAip1−/− testes can efficiently re-colonize recipient testes. This result suggested that disruption of SSC function as a result of architectural changes in the postnatal niche is reversible and that the seAip1−/− mice could serve as a good model to study the underlying mechanisms.

Materials and Methods

Mice. Mice were housed in standard cages in an Assessment And Accreditation of Laboratory Animal Care accredited SPF animal facility on a 12 h light/dark cycle. All animal protocols are approved by the Animal Care and Use Committee of the Model Animal Research Center of Nanjing University, the host for the National Resource Center for Mutant Mice in China. All mice used in this study were of a mixed 129S6 background.

Table 1 Transplantation result

| Experiment   | Control         | seAip1−/−         | Fold change |
|--------------|-----------------|-------------------|-------------|
| Colony number/10^5 THY1+ cells | 1.19 | 3.8 | 3.2 |
| Colony length (mm)/10^5 THY1+ cells | 1.30 | 5.24 | 4 |
| Colony number/10^5 THY1+ cells | 5.96 | 9.23 | 1.55 |
| Colony length (mm)/10^5 THY1+ cells | 10.7 | 13.14 | 1.23 |
| Colony number/10^5 THY1+ cells | 18.67 | 27.64 | 1.48 |
| Colony length (mm)/10^5 THY1+ cells | 31.3 | 57.25 | 1.83 |

The total number of colonies from three to six different recipient testes was analyzed for each group.

Histology and immunofluorescence. Testes were fixed with 4% paraformaldehyde, embedded in paraffin and transversely sectioned at a thickness of 5 μm. Tissue sections were stained with HE to examine histology. Immunofluorescence was carried out as previously described. Sections were counterstained with DAPI (Sigma-Aldrich, Saint-Quentin Fallavier, France), mounted in vectashield aqueous medium (Vector Laboratories, Inc., Burlingame, CA, USA), and analyzed using Olympus FV1000 confocal microscope. Primary antibodies used are: rabbit-anti-SOX9 (The original concentration is 200 μg/ml, 1:50), mouse-anti-PLZF (200 μg/ml, 1:200), mouse-anti-GATA4 (200 μg/ml, 1:200), rabbit-anti-PLZF (200 μg/ml, 1:200), mouse-anti-OCT4 (200 μg/ml, 1:100) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), sc-20905, sc-28319, sc-81178, sc-25310, sc-22839, sc-5279, rabbit-anti-MVH (0.8 mg/ml, 1:300), rabbit-anti-STR8 (0.8 mg/ml, 1:300) (Abcam, Cambridge, UK; Ab13840, Ab49620), Rat-anti-Ki67 (0.74 mg/ml, 1:200) (Dako, Glostrup, Denmark; M7249), rabbit-anti-Cleaved caspase-3 (79 μg/ml, 1:200) (Cell Signaling Technology Inc.; #9661), mouse-anti-E-cadherin (250 μg/ml, 1:200), mouse-anti-N-cadherin (250 μg/ml, 1:200) (BD Biosciences, San Jose, CA, USA; 610182, 610920), rat-anti-Vimentin (100 μg/ml, 1:100) (EMD Millipore, MA91997; Bilieric, MA, USA). Cy3- Cy5- and FITC-conjugated antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA; 115-165-146 (1 mg/ml, 1:200), 111-165-003 (0.75 mg/ml, 1:200), 715-175-150 (0.25 mg/ml, 1:200), 114-095-146 (0.75 mg/ml, 1:200), 111-095-144 (0.75 mg/ml, 1:200)) and AlexaFluor 488-conjugated antibodies (Invitrogen, Cergy Pontoise, France, A21208 (2 mg/ml, 1:200)) were used as secondary antibodies. F-actin was counterstained with DAPI (Sigma-Aldrich, Saint-Quentin Fallavier, France), mounted in vectashield aqueous medium (Vector Laboratories, Inc., Burlingame, CA, USA), and analyzed using Olympus FV1000 confocal microscope. Primary antibodies used are: rabbit-anti-SOX9 (The original concentration is 200 μg/ml, 1:50), mouse-anti-PLZF (200 μg/ml, 1:200), mouse-anti-GATA4 (200 μg/ml, 1:200), rabbit-anti-PLZF (200 μg/ml, 1:200), mouse-anti-OCT4 (200 μg/ml, 1:100) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), sc-20905, sc-28319, sc-81178, sc-25310, sc-22839, sc-5279, rabbit-anti-MVH (0.8 mg/ml, 1:300), rabbit-anti-STR8 (0.8 mg/ml, 1:300) (Abcam, Cambridge, UK; Ab13840, Ab49620), Rat-anti-Ki67 (0.74 mg/ml, 1:200) (Dako, Glostrup, Denmark; M7249), rabbit-anti-Cleaved caspase-3 (79 μg/ml, 1:200) (Cell Signaling Technology Inc.; #9661), mouse-anti-E-cadherin (250 μg/ml, 1:200), mouse-anti-N-cadherin (250 μg/ml, 1:200) (BD Biosciences, San Jose, CA, USA; 610182, 610920), rat-anti-Vimentin (100 μg/ml, 1:100) (EMD Millipore, MA91997; Bilieric, MA, USA). Cy3- Cy5- and FITC-conjugated antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA; 115-165-146 (1 mg/ml, 1:200), 111-165-003 (0.75 mg/ml, 1:200), 715-175-150 (0.25 mg/ml, 1:200), 114-095-146 (0.75 mg/ml, 1:200), 111-095-144 (0.75 mg/ml, 1:200)) and AlexaFluor 488-conjugated antibodies (Invitrogen, Cergy Pontoise, France, A21208 (2 mg/ml, 1:200)) were used as secondary antibodies. F-actin was counterstained with DAPI (Sigma-Aldrich, Saint-Quentin Fallavier, France)
Flow cytometry. The experiment was carried out as previously described. 45 In brief, the single-cell suspension of the P9 testes cells derived from two-step enzymatic digestion were incubated with the Anti-Mouse CD117 (c-Kit) APC-eFluor 780 antibody (0.125 μg/test) (eBioscience, Inc., San Diego, CA, USA). For the PLZF FACS, the P9 testes cell suspensions were first fixed and washed using the transcription factor buffer set (BD Biosciences, 562574) before incubated with PLZF (D-9) (0.2 μg/test) (sc-28319), then the Cy5-conjugated Goat-anti mouse antibody (Jackson ImmunoResearch Laboratories, 715-175-150 (0.5 μg/ml, 1:200)) were incubated after washing by washperm provided in the buffer set. The stained cells were analyzed by FACS Calibur (BD Biosciences).

**In vitro proliferation assay.** After initial seeding density at 2 x 10^5 cell/well in 12-well plates, cultured THY1+ cells with a typical clump-forming morphology after 6 days culture were gently blown off from STO feeder layer and collected into a 15-mlx conical tube. After rinse and centrifugation, cells were dissociated with Trypsin-EDTA to achieve a single-cell suspension. Following three washes in HBSS buffer, cells were concentrated and counted with Hemocytometer under microscope.

Transplantation assay. Apo1^fl/fl:Amh^cre Rosea26-mTmG and Apo1^fl/fl: Rosea26-mTmG mice were used as the mutant and control donors. MACS THY1+ cells were isolated from 12 testes of six mice at the age of P7–P8. 2.5 x 10^5 THY1+ cells were transplanted into one C57BL/6 J × 129sv F1 recipient testis, which were treated with busulfan at 60 mg/kg, 4 days before busulfan treatment. The colony number was analyzed under the NIKON ECLIPSE E800 fluorescence microscopy.

RNA extraction and RT-PCR. The gene Apo1^-/-, Apo1^+/+ and control mouse testes were stripped of the tunica albuginea, placed in liquid nitrogen. Total RNAs were extracted using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Three microgram of RNA was used for First-strand cDNA synthesis using RevertAid First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON, USA). To quantify mRNA expression using RT-PCR, comparisons were made by normalizing the expression to that of glyceraldehyde-3-phosphate dehydrogenase (Gapdh) – mRNA expression was determined using the BCA Protein Assay kit (23255, Pierce, Chicago, IL, USA). In total, 2–3 μg of protein extracts were analyzed by immunoblotting with the following primary antibodies: rabbit-anti-AIP1 (22 μg/150 μl, 1:500) (Protein tech group, Chicago, IL, USA; 13676-1-AP), mouse-anti-GAPDH (100 μg/ml, 1:1000) (sc-23223), mouse-anti-PLZF (D-9) (250 μg/ml, 1:1000) (sc-28319). HRP-conjugated secondary antibodies were used (Pierce, 31430 (0.8 mg/ml, 1:2000), 31460 (0.8 mg/ml, 1:2000)).

**Conflict of Interest** The authors declare no conflict of interest.

**Acknowledgements.** This work is supported by the grant from NSFC grants and 973 Program of China to X.H. (31471400, 2011CB944300) and NSFC grants to JC (31071219, 2011CB944300). Acknowledgements

1. Oatley JM, Birnster RL. The germ line stem cell niche in mammalian testes. Physiol Rev 2012; 92: 577–595.
2. Saitou M, Yamaji M. Primordial germ cells in mice. Cold Spring Harb Perspect Biol 2012: 4: 1–19.
3. Haukka C, Clermont Y. Evolution of gonocytes in the rat testis during late embryonic and early postnatal life. Arch Anat Histol Embryol 1969: 1: 341–344.
4. Clermont Y, Peray B. Quantitative study of the cell population of the seminiferous tubules in immature rats. Am J Anat 1957; 100: 241–257.
5. Coby M. Gonocytes, the forgotten cells of the germ cell lineage. Birth Defects Res C Embryo Today 2009; 87: 1–26.
6. Belve AR, Cavicchia JC, Millette CF, Deborah AO, Bhatnagar YM, Martin D. Spermatogenic cells of the papuepital mouse: isolation and morphological characterization. J Cell Biol 1977; 74: 68–85.
7. Kim S, Bardwell VJ, Zarkower D. Cell type-autonomous and non-autonomous requirements for Dmrt1 in postnatal testis development. Dev Biol 2007; 307: 314–327.
8. Nagano R, Tabata S, Nakanishi Y, Onaka S, Kurohmaru M, Hayashi Y. Reproliferation and relocation of mouse male germ cells (gonocytes) during prespermatogenesis. Anat Rec 2000; 258: 210–220.
9. McLean DJ, Friel PJ, Johnston DS, Griswold MD. Characterization of spermatogonial stem cell maturation and differentiation in neonatal mice. Biol Reprod 2003; 69: 2085–2091.
10. Tokuda M, Kadokawa Y, Kurahashi H, Menouche D, Takahashi CDH1 is a specific marker for undifferentiated spermatogonia in mouse testes. Biol Reprod 2007; 76: 130–141.
11. Tokunova EN, Malashicheva AB, Chikhirzhina EV, Kostyleva EI, Zeng W, Luo J et al. E-cadherin is a novel surface marker of spermatogonial stem cells derived from two-step transplantation. Cell Death and Disease 2012; 3: 533–542.
12. Palombi F, Salanova M, Tarone G, Farini D, Stefanini M. Distribution of beta 1 integrin subunit during gonocyte migration and SSC niche architecture. Dev Dyn 2008; 237: 2085–2091.
13. Kanatsu-Shinohara M, Takehashi M, Takashima S, Lee J, Morimoto H, Chuma S et al. E-cadherin as a novel surface marker of spermatogonial stem cells. Biol Reprod 2009; 81: 212–218.
14. Newton SC, Blaschuk OW, Millette CF. N-cadherin mediates Sertoli cell-spermatogenic cell adhesion. Dev Dyn 1993; 197: 1–13.
15. Kanatsu-Shinohara M, Takashima S, Lee J, Morimoto H, Chuma S et al. Homing of mouse spermatogonial stem cells to germine niche depends on beta1-integrin. Cell Stem Cell 2008; 3: 533–542.
16. Inaba M, Yuan H, Salzmann V, Fuller MT, Yamashita YM. E-cadherin is required for gonocyte migration and SSC niche architecture. Cell Stem Cell 2008; 542.
17. Palombi F, Salanova M, Tarone G, Farini D, Stefanini M. Distribution of beta 1 integrin subunit during gonocyte migration and SSC niche architecture. Dev Dyn 2008; 237: 2085–2091.
18. Chu D, Pan H, Wan P, Wu J, Luo J, Zhu H et al. AIP1 acts with cofilin to control actin dynamics during epithelial morphogenesis. Development 2012; 139: 3581–3571.
19. Rodal AA, Tetreault JW, Lappalainen P, Drubin DG, Amberg DC. Aip1p interacts with cofilin protein 1 (XAip1) enhances cofilin fragmentation of filaments by capping filament ends. J Biol Chem 2002; 277: 43011–43016.
22. Ren N, Charton J, Adler PN. The flare gene, which encodes the AIP1 protein of Drosophila, functions to regulate F-actin disassembly in pupal epidermal cells. Genetics 2007; 176: 2223–2234.

23. Ono S. The Caenorhabditis elegans uno-78 gene encodes a homologue of actin-interacting protein 1 required for organized assembly of muscle actin filaments. J Cell Biol 2001; 152: 1313–1320.

24. Kile BT, Panopoulos AD, Stitzaker RA, Hacking DF, Tahtamouni LH, Willson TA et al. Mutations in the cofillin partner Apl1/Wdr1 cause autoinflammatory disease and macrothrombocytopenia. Blood 2007; 110: 2371–2380.

25. Kurokawa S, Kojima Y, Misuno K, Nakane A, Hayashi Y, Kohn K. Effect of epidermal growth factor on spermatogenesis in the cryptorchid rat. J Urol 2005; 174: 2415–2419.

26. Ohta H, Tsumida K, Dohmae K, Nishimune Y. Regulation of proliferation and differentiation in spermatogonial stem cells: the role of c-kit and its ligand SF. Development 2000; 127: 2125–2131.

27. Brannan CI, Bedell MA, Resnick JL, Epping JJ, Handel MA, Williams DE et al. Developmental abnormalities in Steel17H mice result from a splicing defect in the steel factor cytoplasmic tail. Genes Dev 1992; 6: 1832–1842.

28. Nakano RM, Braun RE. Hormonal regulation of spermatogenesis. Int J Androl 2004; 27: 335–342.

29. Petersen C, Soder O. The sertoli cell—a hormonal target and ‘speru’ nurse for germ cells that determines testicular size. Horm Res 2006; 66: 153–161.

30. Ogawa T, Areshchag JM, Avarbock MR, Brinster RL. Transplantation of testes germinal cells into mouse seminiferous tubules. Int J Dev Biol 1997; 41: 111–122.

31. Takashima S, Kanatsu-Shinohara M, Tanaka T, Takehashi M, Morimoto H, Shinohara T. Rac mediates mouse spermatogonial stem cell homing to germine niches by regulating transmigration through the blood-testis barrier. Cell Stem Cell 2011; 9: 465–473.

32. Reding SC, Stepnoski AL, Cloninger EW, Oatley JM. THY1 is a conserved marker of undifferentiated spermatogonial cells in the pre-pubertal bull testes. Reproduction 2010; 139: 893–903.

33. Guo Y, Hsi Y, Gong Y, Li Z, He Z. Characterization, isolation, and culture of mouse and human spermatogonial stem cells. J Cell Physiol 2014; 229: 407–413.

34. Bussell MJ, Galloway RM, Fentiman SJ, Dragani TA, Sykes-Brown WJ, Clarke NG et al. Expression and regulation of transcription factors GA-TA-4 and GA-TA-6 in developing testis. Dev Biol 2008; 315: 364–375.

35. Prabhu SM, Meistrich ML, McLaughlin EA, Roman SD, Warne S, Mendis S. Role of c-kit in spermatogenesis: identification of spermatogonia as a specific site of c-kit expression and function. J Cell Sci 1991; 98: 459–470.

36. Tanaka SS, Toyooka Y, Akasu R, Katoh-Fukui Y, Nakahara Y, Suzuki R et al. Expression and regulation of transcription factors GATA-4 and GATA-6 in developing testis. J Cell Sci 1991; 98: 459–470.

37. Manova K, Nocka K, Besmer P, Bachvarova RF. Gonadal expression of c-kit encoded at the W locus of the mouse. Development 1993; 110: 1057–1069.

38. Yoshinaga K, Nishikawa S, Ogawa M, Hayashi S, Kunisada T, Fujimoto T et al. Role of c-kit in mouse spermatogenesis: identification of spermatogonia as a specific site of c-kit expression and function. Development 1991; 113: 469–475.

39. Zhou Q, Li Y, Re J, Friel P, Mitchell D, Eaves RM et al. Expression of stimulated retinoic acid gene 8 (Strahl) and maturation of murine gonocytes and spermatogonia induced by retinoic acid in vitro. Biol Reprod 2008; 78: 537–545.

40. Aoyama K, Aihara H, Kishida K, Koyama M, Kashiwagi K et al. Sertoli cells promote gonocyte migration and SSC niche architecture in vitro. Dev Biol 2009; 329: 281–292.

41. Tani J, Horikoshi N, Otsuka T, Saito J, Kanayama M, Maeda J et al. C-kit silencing demonstrates essential functional roles for actin disassembly during myocardial growth and maintenance in mice. Am J Pathol 2014; 184: 1967–1980.

42. Filipponi D, Hobbs RM, Otolski S, Ross P, Jannin EA, Pandolfi PP et al. Regulation of c-kit expression in spermatogonial stem cells. Mol Cell Biol 2000; 20: 4384–4393.

43. Prabhu SM, Meistrich ML, McLaughin EA, Roman SD, Warne S, Mendis S et al. Expression of c-kit receptor mRNA and protein in the developing, adult and irradiated rodent testis. Reproduction 2000; 119: 489–499.

44. Manova K, Nocka K, Besmer P, Bachvarova RF. Gonadal expression of c-kit encoded at the W locus of the mouse. Development 1990; 110: 1057–1069.

45. Aoyama K, Aihara H, Kishida K, Koyama M, Kashiwagi K, Otsuka T et al. C-kit silencing demonstrates essential functional roles for actin disassembly during myocardial growth and maintenance in mice. Am J Pathol 2014; 184: 1967–1980.

46. Ishii K, Kanatsu-Shinohara M, Toyokori S, Shinohara T, FGF2 mediates mouse spermatogonial stem cell self-renewal via upregulation of Etv5 and Bolb through MAP2K1 activation. Development 2012; 139: 1734–1743.

47. Kurokawa S, Kojima Y, Misuno K, Nakane A, Hayashi Y, Kohn K. Effect of epidermal growth factor on spermatogenesis in the cryptorchid rat. J Urol 2005; 174: 2415–2419.

48. Ohta H, Tsumida K, Dohmae K, Nishimune Y. Regulation of proliferation and differentiation in spermatogonial stem cells: the role of c-kit and its ligand SF. Development 2000; 127: 2125–2131.