Stem Cell Factor/c-kit Up-Regulates Cyclin D3 and Promotes Cell Cycle Progression via the Phosphoinositide 3-Kinase / p70 S6 Kinase Pathway in Spermatogonia

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Running Title: C-kit Signaling via PI3-K in Spermatogonia

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SUMMARY

Stem cell factor (SCF) / c-kit plays an important role in the regulation of hematopoiesis, melanogenesis, and spermatogenesis. In the testis, the SCF/c-kit system is believed to regulate germ cell proliferation, meiosis, and apoptosis. Studies with type A spermatogonia in vivo and in vitro have indicated that SCF induces DNA synthesis and proliferation. However, the signaling pathway for this function of SCF/c-kit has not been elucidated. We now demonstrate that SCF activates phosphoinositide 3-kinase (PI3-K) and p70 S6 kinase (p70S6K) and that rapamycin, a FRAP/mTOR dependent inhibitor of p70S6K, completely inhibited BrdU incorporation induced by SCF in primary cultures of spermatogonia. SCF induced cyclin D3 expression and phosphorylation of the retinoblastoma protein (Rb) through a pathway that is sensitive to both Wortmannin and rapamycin. Furthermore, AKT but not protein kinase C-zeta (PKC-ζ), is used by SCF/c-kit/PI3-K to activate p70S6K. Dominant negative AKT-K179M completely abolished p70S6K phosphorylation induced by the constitutively active PI3-K catalytic subunit p110. Constitutively active v-AKT highly phosphorylated p70S6K, which was totally inhibited by rapamycin. Thus, SCF/c-kit uses a rapamycin sensitive PI3-K/AKT/p70S6K/cyclin D3 pathway to promote spermatogonial cell proliferation.

INTRODUCTION

Spermatogenesis is a complex process of cellular renewal and differentiation that culminates in the production of spermatozoa (1). Type A spermatogonia, the male germ line stem cells, divide by mitosis to yield other type A stem cells as well as the differentiated type A
spermatogonia (2). The differentiated type A proceed through a series of mitotic divisions to produce meiotic spermatocytes. The early spermatocytes enter the first of two meiotic divisions producing round spermatids; the round spermatids then differentiate into late spermatids that are released into the lumen of the seminiferous tubules as spermatozoa. Thus far, the molecular mechanisms and signaling pathways for these events remain largely unknown. It has been suggested that the stem cell factor/c-kit system may play a crucial role in regulating proliferation and differentiation of spermatogonial stem cells (3-5). In the testis, Sertoli cells, the supportive elements within the seminiferous epithelium, produce stem cell factor (6-8). SCF exists in either a soluble form or membrane-bound to Sertoli cells. Type A spermatogonia express the receptor for SCF, c-kit (3,9). Both the ligand and the receptor are expressed in a stage specific manner (10). Studies on the self-renewal and proliferation of spermatogonia have demonstrated that: 1) administration of ACK-2, a monoclonal antibody that blocks the binding of SCF to c-kit, disrupts proliferation of mouse spermatogonia and promotes apoptosis (4); 2) recombinant SCF stimulates the division of isolated primary spermatogonia (6). These studies indicate that SCF/c-kit regulates spermatogonial mitotic progression, a process indispensable for the self-renewal and differentiation of the male germ-line stem cell population. However, the signal pathway triggered by SCF and its downstream cascade to regulate spermatogonial cell mitosis are not known.

Recent studies suggest that PI3-K acts as an immediate downstream molecule of growth factor receptors to mediate mitogenic signaling in cells. The activation of PI3-K is sufficient to drive some types of cells to go through G1/S transition and to proliferate (11). p70S6K mediates PI3-K signaling to the cell cycle machinery leading to proliferation in a variety of cell types.
(12,13). In the present study, we addressed whether the PI3-K signaling pathway is activated by SCF to regulate the proliferation of spermatogonia. We observed that SCF induced the PI3-K/AKT/p70S6K signaling pathway. Rapamycin completely blocked cyclin D3 expression and the BrdU incorporation induced by SCF. Taking these data together, our studies indicted that SCF/c-kit recruits PI3-K/AKT/p70S6K/cyclin D3 to stimulate proliferation of type A spermatogonia.
EXPERIMENTAL PROCEDURES

Cell preparation, Culture, Plasmids, and Transfection—The testes of 5 day-old mouse pups (Balb-c) were removed and digested with a mixture of enzymes that included collagenase, trypsin, and hyaluronidase; then type A spermatogonial cells were isolated from the resultant mixture of testicular cells using a BSA gradient column as described previously (9,14). Isolated spermatogonial cells were cultured in DMEM/F12 (Irvine Scientific) containing 10% horse serum (Biofluids, Inc.) and antibiotics (Sigma). Long-term maintenance of the spermatogonia was achieved by co-culturing the cells with the 15P-1-Sertoli cell line (15). The expanded spermatogonial cells were further purified by differential plating. Plasmids expressing wild type p70S6K and the AKT-K179M were provided by Dr. Xiantao Wang (National Institute of Aging, NIH). pLXSN-v-AKT was provided by Dr. Peter Sabbatini (the Cancer Research Institute, University of California School of Medicine, San Francisco). All transient transfections were performed using LipoFectin reagent following the protocol provided by the manufacturer (GIBCO BRL).

Immunoprecipitation and Immunoblotting—Cells were harvested and lysed with the following buffer (20 mM HEPES pH7.4, 50 mM beta-glycerol phosphate, 2mM EGTA, 1 mM DTT, 10 mM NaF, 1mM Sodium orthovanadate, 1% triton X-100, 10% Glycerol, and protease inhibitors: 2µg/ml leupeptine and 2mM phenylmethylsulfonyl fluoride). To immunoprecipitate c-kit, whole cell lysates containing 100 µg total proteins were incubated with 1µg/ml c-kit polyclonal antibody (Santa Cruz Biotechnology) for 1 hour at 4°C. Immunoprecipitation was facilitated by addition of protein A-Sepharose (Pharmacia Biotech) for 2 hours at 4°C on a
rotating shaker. The immunoprecipitate was washed three times at 4°C with the lysis buffer. Similarly, the p85 regulatory subunit of PI3-K was immunoprecipitated by incubating 300 µg total protein with the monoclonal p85 antibody (Upstate Biotechnology) and protein G (Pharmacia Biotech). The immunoprecipitate was washed three times at 4°C with lysis buffer. The immunoprecipitated proteins were released from beads by boiling and were loaded onto SDS-PAGE. The separated proteins were blotted onto the nitrocellulose membranes.

For immunoblotting, cell lysates were subjected to SDS-PAGE and proteins were transferred to polyvinylidene difluoride membranes. Membranes were blocked with TBS buffer containing 5% fat free milk, and 0.1% Tween 20 for 1 hour, and hybridized with the primary antibodies: anti-phosphotyrosine 4G10 (Upstate Biotechnology), c-kit polyclonal antibody (Santa Cruz Biotechnology), AKT antibody, and phospho-AKT antibodies (New England Biolabs, Inc), p70S6K antibody and phospho-p70S6K antibodies (New England Biolabs, Inc), and Rb antibody and phospho-Rb antibodies (New England Biolabs, Inc), cyclin D3 antibody (Signal Transduction Lab), PKC-ζ antibody (Santa Cruz Biotechnology). After hybridization with secondary antibodies conjugated to horseradish peroxidase, the immunocomplex was detected with the ECL detection reagent (Amersham Life Sciences, Inc).

*BrdU Incorporation Assay*—Freshly isolated type A spermatogonial cells were plated on cover slides with serum free DMEM/F12. The cells were incubated with 50nM rapamycin for 30 minutes, then 100 ng/ml mSCF (R & D Systems, Inc) and 30 µg/ml bromodeoxyuridine BrdU (Sigma) were added to the cells. After 18 hours of culture, the cells were fixed in 70% ethanol.
and stained for BrdU following the protocol provided by the manufacturer (Zymed Laboratories, Inc.).

**PKC-ζ Activity Assay**—Spermatogonia cells were transfected with active p110 and cultured for 48 hours, then the cells were starved in serum free medium for 24 hours. The cells were lysed in 20 mM Tris pH7.5, 10% glycerol, 1% NP-40, 10 mM EDTA, 150 mM NaCl, 20 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium vanadate, 1µg/ml leupeptin, and 1mM phenylmethylsulfonyl fluoride at 4°C. 500 µg total protein were used to immunoprecipitate PKC-ζ by incubating with 1µg anti-PKC-ζ antibody and 50 µl of 50% protein A-Sepharose in a rolling tube overnight at 4°C. Immunoprecipitates were washed three times with lysis buffer containing 0.5 M NaCl, and washed two times with kinase buffer (35 mM Tris pH7.5, 10 mM magnesium chloride, 1 mM EGTA, 2 mM sodium orthovanadate). PKC-ζ activity was assayed in 30 µl of kinase buffer containing 10 µCi [γ-32p]adenosine triphosphate (Amersham) and 4 µg myelin basic protein (MBP) for 30 minutes at room temperature. The reaction was stopped by the addition of sample buffer, and phosphorylated myelin basic protein was resolved by SDS-PAGE.

**Results**

**Stem Cell Factor Induces Tyrosine Phosphorylation of c-kit and the binding of the PI3-K p85 Subunit to c-kit**—The isolated spermatogonial cells were cultured in serum free medium for 8 hours, then treated with 100 ng/ml mSCF (R & D Systems, Inc) for 5 or 10 minutes. 30µg
whole cell lysates were resolved by SDS-PAGE and blotted with phosphotyrosine antibody (4G10). SCF induced tyrosine phosphorylation of a ~120 kDa protein (Figure 1A). To confirm that this protein is c-kit, 100µg lysates of the control and SCF-treated cells (for 10 min), were immunoprecipitated with 1µg/ml c-kit antibody. The immunoprecipitate was resolved by SDS-PAGE and blotted with the 4G10 antibody. The results showed that the 120 kDa protein was immunoprecipitated by the c-kit antibody and was tyrosine-phosphorylated (Figure 1B). Furthermore, 300µg of cell lysates were immunoprecipitated with the PI3-K (1µg/ml) p85 subunit monoclonal antibody and blotted with 4G10 phosphotyrosine antibody. The tyrosine phosphorylated c-kit was co-immunoprecipitated with the p85 subunit of PI3-K (Figure 1C). These results demonstrated that SCF/c-kit triggers PI3-K signaling in spermatogonia and that the PI3-K signaling pathway may be the pathway for SCF to regulate the proliferation of spermatogonial cells.

SCF/c-kit Induces Wortmannin and Rapamycin Sensitive Phosphorylation of p70 S6 Kinase in Spermatogonia—p70S6K is a down stream regulator of the PI3-K signaling pathway. It has emerged as an important regulator of cell growth, playing a positive role during progression through the G1 phase of the cell cycle (12). Thus, we next tested whether p70S6K is also activated by SCF/c-kit through PI3-K in type A spermatogonial cells. Isolated spermatogonial cells were cultured in serum-free medium overnight and then the cells were pretreated with 100 nM Wortmannin (an inhibitor of PI3-K) or 50 nM rapamycin (an mTOR dependent inhibitor of p70S6K) for 30 minutes, then stimulated with 100 ng/ml mSCF (R&D System) for 45 minutes or 6 hours. 40 µg protein extracts of treated cells were resolved by SDS-PAGE and blotted with phospho-p70S6K antibodies including anti-Thr389 and anti-
Thr421/Ser424. As shown in Fig. 2, SCF induced phosphorylation of p70S6K strongly at Thr-389 at both the 45 minute and 6 hour intervals. This phosphorylation was completely inhibited by both Wortmannin and rapamycin. Thus, it is clear that SCF/c-kit activates a rapamycin sensitive PI3-K/p70S6K signaling pathway in spermatogonial cells.

Rapamycin Blocks SCF Induced BrdU Incorporation in Type A Spermatogonia—The above results suggested that SCF/c-kit may use the PI3-K/p70S6K pathway to cause cell cycle progression in spermatogonia. However, in addition to PI3-K/p70S6K, the cell cycle may be regulated by other multiple mitogenic pathways including Ras-p42/p44 MAPK (16) and PI3-K dependent but rapamycin insensitive pathways (17). To confirm that PI3-K/p70S6K, but not other pathways, is recruited by SCF/c-kit to cause spermatogonial cell proliferation, we performed a BrdU incorporation study in isolated mouse spermatogonia treated in the presence or absence of SCF and rapamycin. Cells were cultured in serum-free medium and then pretreated with 50 nM rapamycin for 30 minutes. 100 ng/ml mSCF (R&D System) and 30 µg/ml BrdU were added to the medium. The cells were maintained in culture for another 18 hours. Then the cells were stained for BrdU. SCF induced a 4-fold increase in BrdU positive cells compared to controls. Rapamycin completely blocked BrdU incorporation induced by mSCF (Fig. 3). Thus, the SCF/c-kit induced DNA synthesis in spermatogonia occurs through the rapamycin sensitive PI3-K/p70S6K pathway.

SCF/c-kit Up-regulates Cyclin D3 Expression and Induces Phosphorylation of Rb through the Rapamycin Sensitive Pathway in Spermatogonia—The above experiments suggested that SCF uses the PI3-K/p70S6K signaling pathway to promote the proliferation of
spermatogonial cells. Next, we addressed how SCF/c-kit/PI3-K/p70S6K regulates cell cycle progression in spermatogonial cells. Cell cycle processing through G1/S transition requires the induction of the D-type cyclins. It was suggested that cyclin D3 may play a principal role in regulating the spermatogonial cell cycle through G1/S phase progression (18). To address whether cyclin D3 is regulated by SCF/c-kit/PI3-K signaling in spermatogonial cells, isolated mouse spermatogonial cells were cultured in serum free medium overnight. After pretreatment with Wortmannin (100nM) or rapamycin (50nM) the cells were treated with 100 ng/ml mSCF (R&D System) for 6 hours or 18 hours. 50μg whole lysates of treated cells were resolved by SDS-PAGE and blotted with cyclin D3 antibody. The results indicated that expression of cyclin D3 was down-regulated after serum withdrawal for 18 hours in spermatogonial cells (Fig. 4, lanes 1 and 2); addition of 100 ng/ml mSCF restored this D-type cyclin expression after 6 hours (Fig. 4, lanes 3 and 4). Interestingly, the induction of cyclin D3 expression by mSCF was blocked by either Wortmannin or rapamycin (Fig. 4, lanes 5, 6, 7, and 8). Moreover, the same samples were used to analyze the phosphorylation status of RB with phospho-Rb antibodies including phospho-Rb (Ser780), phospho-Rb (Ser249/252), Phospho-Rb (Thr373), phospho-Rb (Ser795) and phospho-Rb (Ser807/811). Only phospho-Rb (Ser780) gave a strong phosphorylation signal (Fig. 4, top panel). Furthermore, the phosphorylation at Ser780 of Rb was also blocked by both Wortmannin and rapamycin (Fig. 4, lanes 5, 6 7, and 8). These results indicated that SCF increases the expression of cyclin D3 and phosphorylates Rb at Ser780 through the PI3-K/p70S6K signaling pathway.

SCF/c-kit/PI3-K Inducing the Phosphorylation of p70S6K Involves AKT Activity but is Independent of PKC-ζ in Spermatogonia—The signaling between PI3-K and p70S6K is
complex. Recently, PKC-ζ and AKT, two important regulators of cell growth, have been demonstrated to mediate the PI3-K signaling to p70S6K. A study with neonatal rat gonocytes, which are the precursors of type A spermatogonia and express c-kit (19), demonstrated that PKC-ζ is co-expressed with PI3-K (20). Western blotting showed that PKC-ζ is expressed in mouse spermatogonia (Fig. 5A). The constitutively active catalytic p110 subunit of PI3-K activated PKC-ζ, and this activity was suppressed to basal levels by 100 µM PKC-ζ pseudosubstrate, an inhibitor of PKC-ζ, (Fig. 5B). To test whether PKC-ζ mediates the signal between PI3-K and p70S6K in spermatogonial cells, the cells were cotransfected with the constitutively active catalytic p110 subunit of PI3-K and p70S6K in the presence of 100 µM pseudosubstrate peptide. 20µg total protein were resolved by SDS-PAGE and blotted with p-p70S6K (Thr389). The results showed that the pseudosubstrate peptide did not inhibit the phosphorylation of p70S6K induced by the active p110 catalytic subunit (Fig. 5C,D). Thus, PKC-ζ is not involved in the SCF/c-kit/PI3-K signaling to activate p70S6K in spermatogonial cells.

AKT acts as a downstream kinase of PI3-K, and its activity is stimulated by a variety of growth factors and insulin via a Wortmannin sensitive pathway (21,22). Activated AKT phosphorylates p70S6K through an indirect pathway, which is not completely understood. To test whether AKT mediates SCF/c-kit/PI3-K signaling to p70S6K in spermatogonial cells, we carried out an experiment analyzing AKT activity and its affect on p70S6K phosphorylation by SCF/c-kit/PI3-K in spermatogonia. Isolated mouse spermatogonia cells were serum-starved for 8 hrs, then the cells were pretreated or untreated with 100 nM Wortmannin for 20 minutes, then they were stimulated with 100 ng/ml mSCF for 30 minutes. 50µg whole cell lysates were
resolved by SDS-PAGE and blotted with phospho-AKT (Ser473) antibody. As shown in Fig. 6A, phosphorylation of AKT was induced by SCF through a Wortmannin sensitive pathway.

We cotransfected the spermatogonial cells with p70S6K plasmid and constitutively active AKT (pLXSN-v-AKT) to determine if active AKT induces the phosphorylation of p70S6K. After 48 hours transfection, cells were cultured in serum free medium, with or without 50 nM rapamycin, for 24 hours, then harvested for immunoblotting. 20 µg total proteins were resolved by 10% SDS-PAGE and blotted with p-p70S6K (Thr389). p70S6K was highly phosphorylated by v-AKT and rapamycin completely abolished this function of v-AKT (Fig. 6B). We next cotransfected the spermatogonia with the dominant negative form of AKT-K179M, p70S6K, and constitutively active p110. After 48 hours transfection, cells were cultured in serum free medium for 24 hours, then they were harvested for immunoblotting. 20 µg total proteins were resolved by 10% SDS-PAGE and blotted with p-p70S6K (Thr389). AKT-K179M markedly inhibited p70S6K phosphorylation induced by the constitutively active catalytic subunit p110 of PI3-K (Fig. 6C,D). These results suggest that AKT is the principal positive regulator that mediates the signaling of SCF/c-kit/PI3-K to activate p70S6K and that AKT activates p70S6K via FRAP/mTOR kinase in spermatogonia.
DISCUSSION

The molecular mechanisms regulating spermatogonial proliferation by stem cell factor largely remain unknown. We now demonstrate for the first time that the SCF/c-kit system up-regulates cyclin D3 and promotes cell cycle progression in spermatogonia through a rapamycin sensitive PI3-K/AKT/p70S6K pathway.

*c-kit* belongs to the PDGFR family of receptor tyrosine kinases (23). It plays a crucial role in regulating haemopoiesis, melanogenesis, and spermatogenesis. In the testes, *c-kit* is predominately expressed in type A spermatogonial cells (3,9). In response to its ligand, stem cell factor (SCF), *c-kit* may trigger multiple signaling pathways to regulate the proliferation and/or differentiation of the spermatogonia. However, the signaling pathways induced by *c-kit* and how these signals are linked to spermatogonial cell renewal and/or differentiation are poorly understood. Our data indicated that SCF induced the binding of the PI3-K p85 subunit to *c-kit*. p70S6K is phosphorylated on Thr-389 by SCF/c-kit through the Wortmannin and rapamycin sensitive pathway in this type of cell. Cotransfection experiments showed that constitutively active p110 of PI3-K phosphorylates p70S6K in spermatogonial cells. These data demonstrate that SCF/c-kit recruits the PI3-K /mTOR pathway to activate p70S6K; the latter interacts with the cell cycle machinery to induce cell proliferation.

The cell cycle through the G1/S checkpoint is regulated by multiple mitogenic signaling pathways including Ras-p42/p44 MAPK (16), PI3-K/p70S6K (24), and the PI3-K dependent but rapamycin insensitive pathway (17). Our BrdU experiment indicated that the SCF/c-kit induced
spermatogonial cell proliferation is completely abolished by rapamycin (Fig. 3). The mechanisms of rapamycin blocking BrdU incorporation induced by SCF is likely caused by the inhibitory function of rapamycin on cyclin D3 expression and phosphorylation of Rb (Fig. 4), both of which are necessary to promote cell cycle progression that is induced by SCF in spermatogonia. The inhibition of rapamycin on cyclin D3 expression and phosphorylation of Rb is consistent with its inhibition of p70S6K phosphorylation by SCF. The SCF induced cyclin D3 expression and Rb phosphorylation are also sensitive to Wortmannin, the inhibitor of PI3-K. Thus, the function of PI3-K to up-regulate cyclin D3 expression and phosphorylate Rb is mediated by p70S6K; SCF/c-kit induces the rapamycin sensitive PI3-K/p70S6K/cyclin D3 pathway in regulating the cell cycle progression and growth in spermatogonial cells.

During the cell cycle through the G1 phase, Rb is phosphorylated at multiple sites, including Ser249/252, Ser780, Ser795, Ser807/811, and Thr373, by cyclin D/cdk4 (cdk6) and cyclin E/cdk2. Our data demonstrated that in regulating spermatogonial cell proliferation, SCF mainly induces the phosphorylation of Rb at Ser780, which is phosphorylated only by cyclin D/cdk (25). Thus, it is suggested that cyclin D3 is the early response gene and the possible main target for SCF that drives G1/S phase progression of spermatogonial cells. This result is consistent with the observation that cyclin D3-associated kinase activity was detected in immature, but not adult testes, and that cyclin D3 plays an important role in regulating the self-renewal of male germ stem cell (18).

Both PKC-ζ and AKT, downstream kinases of PI3-K signaling, are highly expressed in spermatogonia. Although PI3-K activated PKC-ζ, blocking of PKC-ζ did not suppress the
phosphorylation of p70S6K induced by the constitutively active PI3-K catalytic subunit p110 (Fig. 5). Thus, PKC-ζ is not involved in PI3-K signaling to activate p70S6K in spermatogonia. AKT is strongly phosphorylated by SCF/c-Kit/PI3-K. AKT is strongly phosphorylated by SCF/c-Kit/PI3-K. Cotransfection studies in mouse type A spermatogonial cells showed that constitutively active AKT induced phosphorylation of p70S6K and dominant negative AKT-K179M completely inhibited phosphorylation of p70S6K induced by the constitutively active PI3-K catalytic subunit, p110. Rapamycin totally abolished phosphorylation of p70S6K induced by constitutively active AKT (Fig. 6). These results indicate that AKT is the main kinase mediating SCF/c-kit/PI3-K to p70S6K through FRAP/mTOR.

A recent study demonstrated that c-kit induced activation of phosphatidylinositol 3-kinase is essential for spermatogonial proliferation and male fertility (26). Although c-kit is required for normal haematopoiesis, melanogenesis, and gametogenesis; mutations, which disrupt the PI3-K binding to c-kit and impaired PI3-K signaling pathway, only resulted in male sterility due to blockage of the proliferation of spermatogonial cells. There were no apparent haematopoietic or pigmentation defects in the homozygous mutant mice (26). Another recent study that disrupted the binding of PI3-K to c-kit resulted in defective oogenesis and spermatogenesis. The males were sterile due to a block in the early stages of spermatogenesis (27). Thus, PI3-K signaling is particularly important for spermatogonial proliferation and spermatogenesis. Our current data confirm these findings and further elucidate the downstream cascade for SCF/c-kit/PI3-K signaling that regulates spermatogonial proliferation. In summary, upon stimulation with stem cell factor, c-kit recruits the rapamycin sensitive PI3-K/p70S6K pathway to induce cyclin D3 expression and phosphorylation of Rb leading to spermatogonial proliferation. AKT is the main
transducer that links c-kit/PI3-K to p70S6K and is also important for spermatogonial proliferation (Fig. 7). The differential signaling pathway in male germ line stem cells leading to stem cell renewal vs stem cell differentiation remains to be elicited.
REFERENCES


Acknowledgments

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The abbreviations used are: SCF, stem cell factor; PI3-K, phosphoinositide 3-kinase; Rb, retinoblastoma protein; mTOR, mammalian target of rapamycin; BrdU, bromodeoxyuridine; PAGE, polyacrylamide gel electrophoresis; PKC-ζ, protein kinase C-zeta.

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FIG. 1. **SCF induces tyrosine phosphorylation of c-kit and the binding of the PI3-K p85 subunit to c-kit.** A, Isolated mouse spermatogonial cells were serum starved for 8 hours, then treated with 100 ng/ml recombinant mouse SCF (R & D Systems, Inc) for 5 minutes or 10 minutes. 30 µg total protein of treated cells were resolved by 4-12% SDS-PAGE and blotted with phosphotyrosine antibody 4G10 (Upstate Biotechnology). B, 100µg total protein of SCF-treated cells were immunoprecipitated with c-kit antibody, and the immunoprecipitate was blotted with phosphotyrosine antibody 4G10 and c-kit antibody. C, 300 µg total protein of SCF-treated cells were immunoprecipitated with antibody to the p85 subunit of PI3-K and the immunoprecipitate was blotted with phosphotyrosine antibody (Upstate Biotechnology).

FIG. 2. **SCF induces both Wortmannin and rapamycin sensitive phosphorylation of p70 S6 kinase.** Isolated mouse spermatogonial cells were cultured in serum free medium overnight, then treated with either vehicle (DMSO), or 100 nM Wortmannin, or 50 nM rapamycin for 30 minutes, then stimulated with 100 ng/ml SCF for 45 minutes or 6 hours. 40 µg total protein of each culture were resolved by 10% SDS-PAGE and blotted with antibody to phospho-p70S6K(Thr 389) and p70S6K antibody.

FIG. 3. **Rapamycin blocks BrdU incorporation induced by SCF in spermatogonia.** Isolated mouse spermatogonial cells were collected after differentially plating and cultured on serum coated cover slides in serum free DMEM/F12. Cells were untreated or pretreated with 50 nM rapamycin for 30 minutes, then 100 ng/ml mSCF (R & D Systems, Inc) and 30 µg/ml BrdU
(Sigma) were added to the medium. Cells were fixed 18 h later and immunostained with biotinylated anti-BrdU and streptavidin-peroxidase and DAB (Zymed). Data represent mean ± standard deviation of three samples. This is a representative experiment performed independently twice.

FIG. 4. **SCF/c-kit up-regulates cyclin D3 expression and induces phosphorylation of Rb.** Isolated mouse spermatogonial cells were cultured in serum free medium overnight, then switched to fresh medium with 10 % serum (lane 2) or without serum (lanes 1,3,4,5,6,7,8). After 30 minutes pretreatment with 100 nM Wortmannin (lane 5,6) or 50 nM rapamycin (lane 7,8), 100 ng/ml mSCF were added to the medium (lanes 3,4,5,6,7,8). Then the culture was continued for another 6 hours (lanes 3,5,7) or 18 hours (lanes 1,2,4,6,8). 50 µg total protein of cells from each culture were resolved by 4-12% SDS-PAGE and blotted with cyclin D3 antibody; then the same filter was blotted with antibody to phospho-Rb(Ser 780) and Rb antibody (top panel). The same amount of protein from each sample was resolved by 10% SDS-PAGE and blotted with antibody to β-actin (bottom panel).

FIG. 5. **PKC-ζ is not involved in the phosphorylation of p70S6K induced by PI3-K.** A, 50 µg total protein from mouse spermatogonial cells or NIH/3T3 cells were blotted with the antibody to PKC-ζ. B, Spermatogonial cells were transfected with constitutively active p110 of PI3-K and cultured for 48 hours; then the cells were starved in serum free medium with or without 100 µM PKC-ζ pseudosubstrate for 24 hours; cells were lysed, and PKC-ζ was assayed as described under “Experimental Procedures”. C, Mouse spermatogonial cells were transfected with constitutively active p110 plasmid, and cultured for 48 hours. Then 100 µM
pseudosubstrate peptide was added to one of p110 transfected cultures, and all cultures were serum starved for 24 hours. 20 µg total protein of each culture were resolved by 4-12% SDS-PAGE and blotted with antibody to p70S6K (Thr389) (C, top panel) and p70S6K antibody (C, bottom panel). The image intensity of phosphorylation of p70S6K was quantitated with the Sigmagel program and normalized by the Western Blot signal for p70S6K of each loading (C, bottom panel) and is presented as a bar graph (D).

FIG.6. SCF/c-kit/PI3-K activates p70S6K through AKT. A, Isolated mouse spermatogonial cells were serum starved for 8 hours, then untreated or treated with 100 ng/ml mSCF (R & D Systems, Inc) for 30 minutes; 50 µg total protein of each culture were resolved by 10% SDS-PAGE and blotted with antibody to p-AKT(Ser473) (top panel) and AKT antibody (bottom panel). B, Mouse spermatogonial cells were transfected with p70S6K or cotransfected with p70S6K plasmid and constitutively active AKT (pLXSN-v-AKT); after culture for 48 hours, 50nM rapamycin were added to one of the cotransfected cell dishes; all three cultures were serum starved for 24 hours; then 20µg total protein from each culture were resolved by 10% SDS-PAGE and blotted with antibody to p-p70S6K (Thr389) (top panel) and p70S6K antibody (bottom panel). C, Mouse spermatogonial cells were transfected with p70S6K plasmid or cotransfected with p70S6K plasmid and active p110 of PI3-K alone or together with dominant negative AKT-K179M; after culture for 48 hours, cells were serum starved for 24 hours, then 20 µg total protein of each culture were resolved by 10% SDS-PAGE and blotted with antibody to p-p70S6K (Thr389) and p70S6K antibody; the image intensity of phosphorylation of p70S6K was quantitated with the Sigmagel program and normalized by the Western Blot signal for p70S6K of each loading then presented as a bar graph (D).
FIG. 7. *c-kit/PI3-k signaling in spermatogonia.* This model demonstrates that *c-kit/PI3-K* promotes cell cycle progression via the AKT/p70S6K/cyclin D3 pathway.
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Rapamycin
Wortmannin
SCF

IB: p-p70S6K (Thr389)
IB: p70S6K
A  
- - +  Wortmannin  
- + +  SCF  
IB: p-AKT (Ser 473) ab  
IB: AKT ab  

B  
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+ + +  p70S6K  
IB: p-p70S6K (Thr389) ab  
IB: p70S6K ab  

C  
- + +  Active p110  
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IB: p-p70S6K (Thr389) ab  
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Li-Xin Feng, Neelakanta Ravindranath and Martin Dym

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