IGF-1R Signaling Is Essential for the Proliferation of Cultured Mouse Spermatogonial Stem Cells by Promoting the G₂/M Progression of the Cell Cycle

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Culture of mouse spermatogonial stem cells (mSSCs) contributes to understanding the mechanisms of mammalian spermatogenesis. Several key growth factors such as GDNF and FGF2 have been known to be essential for the proliferation of cultured mSSCs. However, additional factors regulating SSC proliferation remain to be identified. In this study, we report that IGF-1R signaling is required for the proliferation of cultured mSSCs by promoting the G₂/M progression of the cell cycle. IGF-1 and its receptor IGF-1R are expressed in cultured mSSCs as well as in isolated Sertoli cells and interstitial cells. Blockage of IGF-1R signaling either by knockdown of IGF-1R or by the IGF-1R-specific inhibitor picropodophyllin (PPP) significantly reduced the proliferation of mSSCs, increased their apoptosis, and impaired their stem cell activity in an insulin-independent manner. PPP treatment of mSSCs blocked the G₂/M progression. In contrast, both GDNF withdrawal and FGF2 signaling blockade decreased the entry of mSSCs into their S phases. Consistently, IGF-1 promoted the G₂/M progression of thymidine-treated mSSCs, which were arrested at G₁/S boundary synchronously; while GDNF and/or FGF2 stimulated their entry into the S phase. Moreover, IGF-1 activated the phosphorylation of AKT but not that of ERK1/2 in mSSCs. These results indicate that IGF-1R signaling stimulates the proliferation of mSSCs using a distinct mechanism from those by GDNF and FGF2, and will contribute to the establishment of a chemically defined culture system.

Introduction

THE LIFE-LONG SPERMATOGENESIS of the mammals is sustained by the continuous proliferation and differentiation of spermatogonial stem cells (SSCs), a sub-population of the undifferentiated type A spermatogonia (*A_{undiff}*) [1,2]. Self-renewal and differentiation of SSCs depends on growth factors from the stem cell niche, which consists of surrounding cells, extracellular matrix, and local vasculatures [3,4]. It is generally recognized that Sertoli cells, which physically interact with SSCs, are the major component of the SSC niche [5]. Other testicular somatic cells, including peritubular myoid cells and interstitial Leydig cells outside of the seminiferous tubules, are also considered parts of the stem cell niche. Notably, testicular vasculature is also regarded as an important component of the SSC niche [6].

GDNF produced by Sertoli cell regulates SSC self-renewal in a paracrine manner. Knockout (KO) of *Gdnf*, even in the heterozygous mutant mice, leads to the depletion of the mSSC pool, while its overexpression results in the hyperplasia of mSSCs, which are unable to differentiate and undergo apoptosis on retinoic acid treatment [7]. Based on

these observations, long-term culture of mSSCs was subsequently established by using medium containing GDNF and other growth factors [8,9]. Thereafter, several other growth factors have been reported to enhance the self-renewal and survival of mSSCs. CSF1 from Leydig and myoid cells enhances mSSC self-renewal in a paracrine manner [10]. NODAL is the first reported autocrine factor that stimulates the proliferation of mSSCs [11]. Cell-extrinsic factor WNT5A supports self-renewal of mSSCs, while WNT3A might promote the proliferation of spermatogonial progenitor cells [5,12]. Work from our laboratory shows that endogenously produced FGF2 is essential for the proliferation and survival of mSSCs [13]. Most of these growth factors are involved in G₁/S regulation by facilitating DNA synthesis [11,13,14].

The insulin-like growth factor (IGF) system plays a critical role in both embryonic and postnatal development and adult physiology. The system consists of three ligands (IGF-I, IGF-II, and insulin), four receptors [IGF-IR, the insulin receptor (IR), the hybrid receptor (IR/IGF-IR), and IGF-IIR], as well as six IGF-binding proteins (IGFBP-1 to 6) [15,16]. Moreover, the system also includes intracellular

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signaling proteins such as the insulin receptor substrate (IRS) family [15,16]. IGF-1 plays an indispensable and pleiotropic role during postnatal growth and tissue remodeling by promoting cell proliferation and protecting cells from apoptosis [15,16]. Some of the *Igf-1*^{-/-} mice die shortly after birth, and the surviving mutants are infertile with delayed development [17]. Moreover, cultures of Sertoli, Leydig, and peritubular cells derived from the immature rats secrete IGF-I and IGF-I binding protein (BP) [18]. Furthermore, Kubota et al. first reported that IGF-1 could increase the SSC activity when used in combination with GDNF [9]. These studies indicate that IGF signaling may be involved in male germ cell proliferation and development.

Growth factors stimulate cell proliferation by regulating the progression of the cell cycle. Stimulated by some growth factors, the quiescent G₀ cells re-enter into G₁ phase, and pass through the subsequent phases [19–21]. Exposure to chemicals, free radicals, or ionizing radiations results in the arrest of the cell cycle at the G₁/S, intra-S, and G₂/M checkpoints [22]. IGF-I promotes DNA synthesis of fibroblasts and facilitates cell cycle progression from G₁ to S phase [23]. In addition, overexpression of mGrb10 α , a physical partner of IGF-1R, inhibits the IGF-1-mediated growth with a delay in the S and G₂ phases of the cell cycle [24]. IGF-1 is also required for G₂ progression in the estradiol-induced mitotic cycle [25]. Moreover, IGF-1R inhibitor picropodophyllin (PPP), which specifically inhibits the kinase activity of IGF-1R [26,27], significantly reduces the growth of malignant cells and induces G₂/M-phase accumulation [27,28].

In this study, we investigated the role of IGF-1 and its receptor IGF-1R in the proliferation of cultured mSSCs. We showed that IGF-1 stimulated the proliferation of mSSCs and differentiating spermatogonia, and maintained the survival of these cells. The mitogenic effect of IGF-1 on mSSCs was mediated by its promotion of the cell cycle through the G₂/M phases, in contrast to the G₁/S promoting effects of GDNF and FGF2. These results indicated that mSSCs require different growth factors in order to progress through different phases of the mitotic cell cycle.

Materials and Methods

mSSC culture

Mouse breeding and use followed standard procedures and were approved by the Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences. F1 pups of DBA/2 and C57BL6 mice were used for mSSC culture. Isolation, enrichment, and culture of mSSCs were performed following protocols developed by Kanatsu-Shinohara et al. and Kubota et al. with some modifications [13,29,30]. Briefly, testes from the 5–7 days postpartum (dpp) mice were digested with 1 mg/mL collagenase I (GIBCO; 17100) and 1 mg/mL DNase I (AppliChem; A3778). The small seminiferous tubule fragments were plated on dishes in mouse embryonic fibroblast (MEF) medium containing 10% fetal bovine serum (FBS), and the undifferentiated spermatogonia were collected with several pipetting after 20 h. Then, the cells were cultured on mitomycin-inactivated MEF feeder cells in MEM α supplemented with other components such as BSA, insulin,

transferrin, and free fatty acids [9,13]. Next, 1% FBS, 20 ng/mL of GDNF, and 5 ng/mL of FGF2 were freshly added to the culture each time the medium was changed. The culture medium components were listed in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/scd). mSSCs were passaged every 4–5 days at the dilution of 1:2–1:4 depending on the size of the clumps, and the medium was changed every 2 days. Contaminated somatic cells were gradually eliminated during the initial three to four passages, and the mSSC culture could be maintained for at least 14 months. When cells were cultured on a laminin (Sigma; L2020)-coated dishes for later experiments, the serum was withdrawn. IGF-1R inhibitor PPP (Calbiochem; 407247) and FGFR inhibitor SU5402 (Calbiochem; 572630) were purchased from Merck Company. Thymidine was purchased from Sigma-Aldrich (T1895).

mSSC transplantation and analysis

mSSCs were infected with lentivirus, which contained the GFP-expressing construct. We conducted an mSSC transplantation experiment to assess the stem cell activity of the mSSC cultures [31,32]. Next, 1×10^8 /mL GFP-labeled mSSCs were microinjected into testis of recipient mice, which were pretreated with busulfan to deplete endogenous spermatogenesis. Appearance of GFP colony in recipient testis represented colonization and spermatogenesis from a single donor-derived GFP-mSSC. We examined the formation of GFP colonies and spermatogenesis at 1–2 months after transplantation. Three months after transplantation, offspring derived from transplanted mSSCs was identified under UV light. For quantification of mSSCs after different treatments during culture, a 10 μ L cell suspension of each sample containing approximately 1×10^5 cells was injected into each testis of the recipient mice. One month after transplantation, the number of GFP colonies was counted. In general, one green patch of at least 0.1 mm in length in the seminiferous tubules that formed 1 month after transplantation is defined as one colony derived from a single mSSC [33]. $n=8$ recipients for DMSO group, and $n=9$ recipients for PPP group; all the quantitative data were from three independent experiments and analyzed by *t*-test.

Immunofluorescence staining and flow cytometry analysis

Immunofluorescence staining and flow cytometry analysis of mSSCs for different proteins were carried out following standard protocols. Briefly, mSSCs were fixed with 4% paraformaldehyde (PFA) for 15 min and permeabilized with 0.1% Triton X-100 for 25 min. After blocking in 5% BSA/PBS for 2 h, the cells were incubated with primary antibody overnight at 4°C. Another aliquot of cells was incubated with isotype IgG from the same resource as the primary antibody. After being washed thrice with PBS, the cells were incubated with FITC/TRITC-conjugated secondary antibody for 1.5 h at room temperature (RT). After three washes, 1 μ g/mL DAPI (Sigma-Aldrich) was used to counterstain the nucleus for 10 min. Fluorescent cells were imaged by a Zeiss LSM 710 Meta confocal microscope (Carl Zeiss) or analyzed using the FACS-Calibur system (BD Biosciences). Antibodies used were listed in Supplementary Table S2.

RNA extraction and reverse transcription-polymerase chain reaction

Total RNA was extracted from the various types of cells using RNeasy Pure Micro Kit (Qiagen). Reverse transcription of purified RNA was performed using Reverse Transcription System (Promega) according to the manufacturer's protocol. Polymerase chain reaction (PCR) was performed following the procedure as previously described [34]. The primer pairs of selected genes were designed and listed in Supplementary Table S3, and PCR products were separated by electrophoresis on 1.2% agarose gels.

Knockdown of *Igf-1r* by siRNA transfection

siRNA molecules specifically targeting mRNA of *Igf-1r* were purchased from GenePharma. The sequences of siRNAs were as follows: siRNA-1 (*Igf-1r*-2325): 5'-GACCCAGU AUGCUGUCUAUtt-3' (sense) and 5'-AUAGACAGCAUA CUGGGUctt-3' (antisense); siRNA-2 (*Igf-1r*-3517): 5'-GC UGCAAGUAUGCGUGAAAtt-3' (sense) and 5'-UUUCAC GCAUACUUGCAGCtt-3' (antisense); siRNA-3 (*Igf-1r*-4316): 5'-GCCCUGAUAUGCUGUUUGAtt-3' (sense) and 5'-UCAACAGCAUAUCAGGGCtt-3' (antisense). The negative control duplex was also provided by the GenePharma, and the sequence was as follows: 5'-UUCUCCGAACGUGU CACGUTT-3' (sense), 5'-ACGUGACACGUUCGGAGAA TT-3' (antisense), which was nonhomologous to any mammal genes and widely used in knockdown experiments [35–38]. mSSCs cultured on laminin-coated dishes were transfected with negative control duplex or siRNAs by Lipofectamine RNAiMAX Reagent (Invitrogen) following the manufacturer's procedure. Cy3-conjugated control oligonucleotide (RIBBIO) was used to determine transfection efficiency. Twenty four hours later, proteins were obtained and the knockdown efficiency was evaluated by western blots. Cell recovery fold (CRF) was calculated 5 days after transfection.

Bromodeoxyuridine incorporation assay

mSSCs were treated with DMSO or PPP for 24 h, and 10 µg/mL bromodeoxyuridine (BrdU; Sigma) was added into culture medium for another 12 h. Then, the cells were collected; fixed with 70% ice-cold ethanol dropwise; and incubated for 20 min at RT. After washes with 0.5% BSA/PBS, the cells were denatured with fresh 2N HCl for 20 min at RT. After several washes, the cells were resuspended with 0.1 M sodium borate (Na₂B₄O₇), pH 8.5, to neutralize any residual acid. Subsequently, the cells were blocked in 5% BSA/PBS for 2 h and later incubated with mouse anti-BrdU antibody (BD Pharmingen) at 4°C overnight. Meanwhile, another aliquot of cells was incubated with mouse isotype IgG, which was used as control to adjust the flow cytometer. The FITC-conjugated anti-mouse IgG was used as the secondary antibody. These cells were stained with DAPI and analyzed using FACS-Calibur system (BD Biosciences).

TUNEL assay

After being treated with PPP for 48 h, the mSSCs were collected and labeled using the DeadEnd™ Fluorometric TUNEL System (Promega) following the manufacturer's protocol. Briefly, the cells were fixed with 4% PFA on ice

for 20 min followed by incubation with 70% ice-cold ethanol for 4 h at –20°C. After a brief wash, the cells were resuspended in Equilibration Buffer for 5 min at RT. Subsequently, the cells were incubated with reaction buffer containing Equilibration Buffer, Nucleotide Mix, and rTdT Enzyme in a water bath for 60 min at 37°C, and resuspended every 15 min. Another aliquot of incubation buffer without rTdT Enzyme was also prepared for the negative control. The reaction was terminated with 20 mM EDTA, and the cells were stained with DAPI and analyzed using the FACS-Calibur system (BD Biosciences).

Propidium iodide staining and cell cycle analysis

The mSSCs were collected and fixed in 70% ice-cold ethanol at 4°C overnight. After several washes with PBS, the cells were stained with 0.02 mg/mL propidium iodide and 0.2 mg/mL RNase at 37°C for 30 min. The cells were analyzed using the FACS-Calibur system (BD Biosciences). Cell cycle phase distributions were analyzed by ModFit software (Verity Software House).

Western blot

Western blot was conducted according to the procedure previously described [13]. Briefly, 4 × 10⁵ mSSCs after different treatments were washed with PBS and lysed using 2 × SDS-PAGE loading buffer. Proteins were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes (WhatmanProtran). Membranes were then blocked with 5% nonfat milk for 1 h at RT, and then incubated overnight with various primary antibodies at 4°C. After several washes with PBST, membranes were incubated with HRP-conjugated secondary antibodies for 2 h at RT. The proteins were detected using the Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific) and imaged by ChemiDoc™ XRS+ system with Image lab™ software (Bio-Rad).

Statistics

All experiments were independently repeated at least thrice. Data are presented as mean ± SEM. Statistical analysis was determined using *t*-test for a single comparison or analysis of variance for a multiple comparison followed by LSD test. All statistical tests were performed using SPSS 13.0 software (SPSS, Inc.).

Results

Expression of IGF-1 and its receptors in mSSCs and testicular somatic cells

Long-term culture of mSSCs does not require the supplement of exogenous IGF-1 [13,29,30]. However, one study by Kubota et al. indicated that inclusion of IGF-1 in culture medium in addition to GDNF, GFRα1, and FGF2 increased the proportion of mSSCs evaluated by the number of colonies established by transplanted mSSCs [9]. We cultured mSSCs in the long term by following procedures established by Shinohara and Brinster laboratories with some modifications (see Supplementary materials and Supplementary Figs. S1 and S2 for details). We reasoned that endogenous IGF-1 might be present from either the serum or

the feeder cells or the mSSCs themselves. Indeed, *Igf-1* mRNA was detected in mSSCs and MEFs. It was also expressed in isolated Sertoli cells (SCs) and interstitial cells (ICs). In contrast, the *Igf-1* mRNA was barely detectable in mouse embryonic stem cells. We also detected the mRNAs of IGF-1 receptor (*Igf-1r*), insulin receptor (*Insr*), and IGF-1R signaling proteins such as insulin receptor substrate 1 and 2 (*Irs1*, *Irs2*) in these cell types. In contrast, as the main endocrine organ of IGF-1, the mouse liver expressed the

mRNA of *Igf-1* but not of *Igf-1r* (Fig. 1A and Supplementary Fig. S3). We next detected the protein expression of IGF-1 and IGF-1R in the cultured mSSCs by immunofluorescent staining. MVH (Mouse Vasa Homolog), a germ cell-specific RNA helicase, is expressed in the cytoplasm of spermatogenic cells, including spermatogonia [39,40]. DAZL (Deleted in azoospermia-like), an RNA-binding protein that is essential for germ cell differentiation, is also expressed in the cytoplasm of premeiotic germ cells [41].

FIG. 1. Expression of IGF-1R signaling genes. **(A)** The reverse transcription-polymerase chain reaction detection of the expression of *Igf-1*, its receptor *Igf-1r*, insulin receptor *Insr*, and insulin receptor substrate *Irs1*, *Irs2* in the liver, embryonic stem cells (ESC), cultured mouse spermatogonial stem cells (mSSCs), mouse embryonic fibroblasts (MEFs), isolated Sertoli cells (SC), and interstitial cells (IC). **(B)** IGF-1 was detected in the cytoplasm of PLZF- or MVH-positive mSSCs. **(C)** IGF-1R protein was localized in the cytoplasm and the nucleus of PLZF-, GFR α 1-, or DAZL-positive mSSCs. Color images available online at www.liebertpub.com/scd

PLZF (Promyelocytic Leukemia Zinc Finger), a transcription factor required for self-renewal of mSSCs, serves as a marker of undifferentiated spermatogonial [42]. As a subunit of GDNF receptor, GFR α 1 is also considered to be expressed in undifferentiated spermatogonial [43,44]. We used antibodies against these marker proteins for their colocalization with IGF-1 and/or IGF-1R. Immunostainings indicated that IGF-1 protein was localized in the cytoplasm of the PLZF- or MVH-positive mSSCs (Fig. 1B). IGF-1R was expressed in both the cytoplasm and the nucleus of PLZF-, GFR α 1-, or DAZL-positive mSSCs (Fig. 1C), which was consistent with the previous studies [45–48], implying that IGF-1R might play a role in transcription regulation in cultured mSSCs. These results suggested that IGF-1R signaling pathways may be involved in regulating the proliferation and/or differentiation of mSSCs both in vitro and in vivo.

IGF-1R signaling pathway is required for the proliferation and stem cell activity of cultured mSSCs

We next examined the effect of IGF-1R signaling on the proliferation of mSSCs by specifically blocking the signaling pathway using an IGF-1R-specific inhibitor PPP, which interferes with the autophosphorylation of IGF-1R [27]. We found that PPP significantly reduced mSSC proliferation in a dose-dependent manner (Fig. 2A and Supplementary Fig. S4A). PPP at concentrations higher than 1 μ M significantly reduced clump formation of mSSCs. Accordingly, we used 1 μ M PPP in the subsequent experiments. Moreover, PPP treatment reduced mSSC proliferation in both the presence and absence of insulin (Supplementary Fig. S4A), suggesting that IGF-1R signaling in mSSCs was independent of the insulin pathway. CRF values, which were the amplification folds of cells during two passages, were then calculated and compared. CRF of 1 μ M PPP-treated cells were significantly decreased (0.16 ± 0.05 -folds, $n=4$) in comparison with untreated cells after 3 days of treatment (2.46 ± 1.58 -folds, $n=4$) (Fig. 2C). In the absence of insulin, the CRFs with and without PPP were 0.15 ± 0.07 -fold and 2.46 ± 0.09 -fold, respectively.

To test whether ligands of IGF-1R were produced by mSSCs, we conducted the blockage experiments using serum-free and feeder-free cultures. mSSCs were cultured on laminin-coated dishes and treated with 1 μ M PPP for 2 days. As shown in Fig. 2B and D, the CRFs of mSSCs with and without PPP treatment were significantly different in an insulin-independent manner. This result indicated that endogenous IGF-1R ligands from mSSCs executed their function in an autocrine manner. To confirm this result, we conducted the proliferation assay by knockdown of *Igf-1r* with specific siRNA oligonucleotides. mSSCs cultured on laminin-coated dishes were transfected with negative control duplex and siRNAs that were specific to IGF-1R. Transfection efficiency was evaluated by the proportion of red fluorescent cells at 24 h after transfection of Cy3-conjugated control oligonucleotides (Supplementary Fig. S4B). Western blot results showed that IGF-1R protein was reduced at 24 h after transfection of either one of the three siRNAs (siRNA1, siRNA2, and siRNA3) (Fig. 2E and Supplementary Fig. S4C), in comparison with that of negative control duplex transfected cells. As expected, a re-

duction in both clump size and cell number was observed in siRNA-treatment cells as compared with those of the negative control transfected cells after 5 days of transfection (Fig. 2F, G).

Stem cells constitute only a subpopulation of the mSSC cultures despite the name “cultured mSSCs” for simplicity. Only stem cells can colonize in recipient seminiferous tubules after transplantation [31,33]. We next tested whether IGF-1R signaling blockade would impair the stem cell activity of cultured mSSCs. GFP-labeled mSSCs (GFP-mSSCs) were derived by infecting mSSCs with lentivirus containing the GFP-expression vector. GFP-mSSCs were first treated with PPP for 36 h, and then, 1×10^5 GFP donor cells were transplanted into each testis of busulfan-pretreated recipient mice. One month later, the recipient testes were analyzed by counting the number of GFP colonies. As shown in Fig. 2H and I, IGF-1R signaling blockage significantly reduced the stem cell activity ($n=8$ recipients for the DMSO group, and $n=9$ recipients for the PPP group). These results demonstrated that IGF-1R signaling is indispensable for the proliferation and stem cell activity of cultured mSSCs.

IGF-1R signaling blockage results in a reduction of cell division and an increase of apoptosis

The reduced proliferation of mSSCs by IGF-1R signaling blockage could be a result of arrested cell cycle or apoptosis or both. We then set out to examine these possibilities. mSSCs were treated with PPP for 24 h, and 10 μ g/mL BrdU was added into the medium for 12 more hours before cells were collected for analysis. BrdU-positive cells were 48% in normal mSSC cultures but were 28% in PPP-treated ones, suggesting that DNA synthesis during the cell cycle was reduced (Fig. 3A, B). Moreover, TUNEL-positive cells were 2.6% in normal cultures but were 56% in PPP-treated ones (Fig. 3C, D). The high percentage of apoptotic cells in PPP-treated mSSCs was consistent with the results that the CRFs were less than 1 when mSSCs were treated with either PPP or siRNAs (Fig. 2C, D, G). These results indicated that IGF-1R signaling is necessary for the proliferation of mSSCs by promoting cell division as well as by maintaining cell survival.

IGF-1R signaling promotes mSSCs to progress through the G₂/M phases of the cell cycle

Ploidy analysis by flow cytometry was conducted to investigate at which phase of the cell cycle PPP-treated mSSCs were arrested. In normal cultures, mSSCs were mostly distributed in the G₁ phase, then in the S phase, and the least in G₂/M phases (Fig. 4A, B). Interestingly, PPP treatment caused mSSCs to be distributed mostly in G₂/M phases (Fig. 4A, B and Supplementary Fig. S5A, B). Furthermore, we used the immunostaining of the mitotic marker phospho-Histone H3 (pH3) to further distinguish between G₂ and M phases. Increased pH3-positive cells after PPP treatment (27.9% vs. 1.6% in control) indicated that a significant portion of mSSCs were arrested at the M phase (Fig. 4C, D). The percentage of G₂ and M phase increased from $7.6\% \pm 1.2\%$, $1.3\% \pm 0.3\%$ in DMSO-treated cells to $37.1\% \pm 7.6\%$, $22.5\% \pm 5.1\%$ in PPP-treated cells, respectively (Fig. 4E). These results indicated that IGF-1R signaling is required for the cell cycle to progress through the G₂/M phases.

FIG. 2. IGF-1R signaling pathway is required for the proliferation and stem cell activity of mSSCs. (A, B) Blockage of IGF-1R signaling by picropodophyllin (PPP) reduced mSSC proliferation on MEF feeder cells (A), laminin-coated dishes (B). (C, D) The values of cell recovery fold (CRF) of 1 μ M PPP-treated cells significantly decreased in comparison with DMSO-treated cells in the presence or absence of insulin. CRFs were evaluated after 3 days of treatment on MEF feeder cells (C) and after 2 days of treatment on a laminin-coated dish (D). (E) Knockdown of *Igf-1r* with specific siRNAs. After transfection with negative control duplex and specific siRNAs against *Igf-1r* for 24 h, the protein was collected and western blots were conducted. (F, G) Knockdown of *Igf-1r* reduced mSSC proliferation of the feeder-free culture at 5 days after transfection as compared with that of the negative control duplex transfected cells; (G) are the quantitative results of (F). (H, I) Blockage of IGF-1R signaling impaired the stem cell activity assessed by transplantation experiments. GFP-labeled mSSCs were treated with DMSO or PPP for 36 h, and then, 1×10^5 GFP donor cells were transplanted into each testis of busulfan-pretreated recipient mice. One month later, the recipient testes were analyzed (H) and the GFP-mSSC colonies (green fragments ≥ 0.1 mm) were counted and plotted in (I). Controls were set using an equal volume of DMSO, which was the solvent of PPP solution. $n=8$ recipients for the DMSO group, and $n=9$ recipients for the PPP group; all the data were from three independent experiments and analyzed by *t*-test. Asterisks represent statistically significant differences (** $P < 0.01$). Significant differences by multiple comparison are marked with different letters ($P < 0.05$). Color images available online at www.liebertpub.com/scd

FIG. 3. Blockage of IGF-1R signaling reduced the mitotic division of mSSCs and increased their apoptosis. (**A, B**) Reduced mSSC proliferation indicated by the decreased percentage of BrdU-labeled cells treated with PPP for 36 h. An aliquot of untreated cells was incubated with mouse isotype IgG, which was used to adjust the flow cytometer. (**C, D**) Increased mSSC apoptosis detected by TUNEL labeled cells treated with PPP for 48 h. To adjust the flow cytometer, an aliquot of untreated cells was incubated in the negative control buffer that was prepared without rTdT Enzyme. Asterisks represent statistically significant differences (** $P < 0.01$). Color images available online at www.liebertpub.com/scd

IGF-1R signaling promotes mSSC proliferation by a distinct mechanism from those by GDNF, FGF2, or insulin

We were interested in knowing how the mechanism of IGF-1's proliferation-promoting effect was different from the mechanisms of GDNF and FGF2, both of which are reported essential factors for the in vitro proliferation of mSSCs. In contrast to the observations made when IGF-1R signaling was blocked, either the withdrawal of GDNF from mSSC culture or the blockage of FGF2 signaling by FGFR inhibitor SU5402 reduced the progression of mSSCs from G₁ to S phases (Fig. 5A, B and Supplementary Fig. S6A, B). To further confirm this difference, we first used thymidine to synchronize mSSCs at the G₁/S boundary (Supplementary Fig. S6C), and then tested whether the addition of different factors would result in different distributions of the mSSCs in the cell cycle (Fig. 5C). Addition of GDNF and/or FGF2 to synchronized mSSCs resulted in a rapid entry of the S phase of these cells, while IGF-1 in combination with GDNF or FGF2 or both promoted the cells to progress to the S and the G₂/M phases significantly than control cells (Fig. 5D). We next assessed whether IGF-1 could activate AKT and ERK1/2 pathway in cultured mSSCs. IGF-1 and/or insulin were added back to the medium of serum-free and feeder-free mSSC culture that was growth factor starved for 3 days, and cell lysates were prepared 30 min later to examine the phosphorylation of AKT and ERK1/2. Both IGF-

1 and insulin could upregulate the phosphorylation of AKT, but not that of ERK1/2 in mSSCs. However, GDNF and FGF2 could activate the phosphorylation of AKT as well as of ERK (Fig. 6A, B). If IGF-1 and insulin activate the same signaling pathway(s), the negative effect of IGF-1R signaling blockage might be rescued by an excess amount of insulin. However, insulin of concentrations from 20 μ g/mL to 200 μ g/mL failed to do so (Supplementary Fig. S6D). Therefore, the effects of IGF-1 and insulin on mSSC culture are different although their regulations on the phosphorylation of AKT and ERK are similar. Collectively, these results indicated that IGF-1 promotes the proliferation of cultured mSSCs using mechanisms that are different from those by GDNF, FGF2, or insulin.

IGF-1R signaling pathway is required for the proliferation of differentiating spermatogonia

We were interested in whether IGF-1R signaling had an effect on the proliferation of differentiating spermatogonia. mSSCs undergo continuous proliferation on mitomycin-inactivated Sertoli cells and begin to express c-KIT (Fig. 7A), a differentiating spermatogonia marker [49,50]. When these cells were treated with PPP for 2 days, c-KIT-positive differentiating spermatogonia significantly decreased compared with cells with DMSO treatment (Fig. 7B). TUNEL assay further demonstrated that apoptosis cells significantly increased after PPP treatment (Fig. 7C and Supplementary

FIG. 4. Blockage of IGF-1R signaling resulted in mSSCs arrested in G₂/M phases. (A, B) mSSCs were treated with DMSO or 1, 2.5 μM PPP for 48 h, and the cell cycle phases were analyzed by propidium iodide staining. The percentages of mSSCs at different cell cycle phases were analyzed by ModFit software (B). (C, D) Mitotic cells marked by pH3 increased when mSSCs were treated with PPP for 24 h. An aliquot of untreated cells was incubated with rabbit isotype IgG, which was used to adjust the flow cytometer. (E) Cell cycle distributions showed that the cell cycle was blocked at G₂ and M phases on the blockage of the IGF-1R signaling by PPP for 24 h. Asterisks represent statistically significant differences (***P* < 0.01). Color images available online at www.liebertpub.com/scd

Fig. S7A). In addition, cell cycle analysis showed that the percentage of G₂/M phases cells increased (Fig. 7D and Supplementary Fig. S7B), suggesting that IGF-1R signaling pathway also regulates the differentiating proliferation of spermatogonia.

Discussion

The establishment of a long-term culture system of SSCs is critical for studying mammalian spermatogenesis and for medical purposes. Many factors essential for SSC proliferation remain to be identified despite the fact that several key factors such as GDNF and FGF2 have been characterized. In this study, we report that IGF-1R signaling is essential for the in vitro proliferation of mSSCs, and that IGF-1 is probably one of the activating ligands. Although all these

growth factors stimulate the mitotic division and promote the survival of mSSCs, the molecular mechanisms utilized are different. For the first time, we show that IGF-1R signaling promotes mSSCs to progress through the G₂/M phases of the cell cycle. These discoveries may contribute to the final establishment of a chemically defined mSSC culture system.

It is challenging to identify essential factors for the in vitro proliferation of mSSCs, although mSSCs can be easily propagated for a long term. This is because the optimal culture system uses MEF feeder cells and low-concentration FBS, which are sources of multiple unidentified factors. A study showed that mSSCs can be cultured in feeder-free and serum-free condition [51]. However, high concentrations of serum product fetuin and BSA were used in this system and contaminated factors cannot be readily identified. The first

FIG. 5. IGF-1 played a distinct role in maintaining the in vitro proliferation of mSSCs from GDNF, FGF2. (A, B) Cell cycle analysis of mSSCs at 2 days after the withdrawal of GDNF (A) or the addition of the FGFR inhibitor SU5402 (B). (C) The scheme of cell cycle synchronization by thymidine and the re-stimulation by growth factors. (D) The G₁/S-arrested mSSCs were re-stimulated with GDNF, FGF2, and IGF-1 for 2 days, and cell cycles were analyzed. Double asterisks represent statistically significant differences ($P < 0.01$). Values with different lowercase letters were significantly different ($P < 0.05$). Color images available online at www.liebertpub.com/scd

FIG. 6. IGF-1 activates the phosphorylation of AKT. (A) Both IGF-1 and insulin could activate the phosphorylation of AKT, but not that of ERK1/2 in mSSCs. mSSCs were serum- and growth factor starved for 3 days, and re-stimulated with insulin and/or IGF-1 for 30 min. The starved cells were also restimulated with GDNF and FGF2 that served as a positive control. Cell lysates were prepared, and western blots were conducted. GAPDH was used as an internal control. (B) The p-AKT and p-ERK1/2 protein levels of different treatments were quantified by densitometry. Double asterisks denote statistically significant differences ($P < 0.01$). NS, no significance. Color images available online at www.liebertpub.com/scd

FIG. 7. Effect of IGF-1R on the proliferation of differentiating spermatogonia. **(A)** mSSCs were cultured on mitomycin-inactivated Sertoli cells and treated with DMSO or PPP. Two days later, the proliferation of differentiating spermatogonia (c-KIT-positive cells) was significantly decreased by PPP treatment in comparison with that of DMSO-treated cells. **(B)** Quantification of differentiating spermatogonia with DMSO or PPP treatments. **(C)** TUNEL assay showed that apoptotic cells were significantly increased after PPP treatment for 2 days. **(D)** After blockage of IGF-1R signaling by PPP for 2 days, the percentage of G₁ and S phases cells was decreased, while that of G₂/M phase cells was significantly increased. *Double asterisks* denote statistically significant differences ($P < 0.01$). Color images available online at www.liebertpub.com/scd

reported essential factor for mSSC in vivo and in vitro proliferation was GDNF [7–9]. It was identified through mouse gene knockout and transgenic expression. Its essentiality for mSSC in vitro proliferation was easily confirmed, because neither the MEFs nor mSSCs themselves produce this factor. The second identified essential factor for the in vitro proliferation of mSSCs was FGF2, which is produced by cultured mSSCs but not by MEFs. Its essentiality was demonstrated by blocking its signaling using a receptor-specific inhibitor or RNA interference of FGF2 [13]. Although other factors such as CSF, NODAL, WNT3A, and WNT5A have been shown to enhance the proliferation of cultured mSSCs, their essentialities have not been demonstrated [5,10–12]. In this study, we showed that IGF-1R signaling, probably activated by its ligand IGF-1 produced by both mSSCs and MEFs, was a third essential factor for the in vitro proliferation of mSSCs.

IGF-1R is expressed in multiple cell types during development and in adult animals, and plays important roles in controlling the proliferation of these cells. Homozygous *Igf-1r* Knockout mice die immediately after birth, and their body weight is only 45% of normal [52]. Similarly, most of the *Igf-1*^{-/-} mice die after birth. Some surviving *Igf-1*^{-/-} mutants are infertile and exhibit delayed development [17]. It had been known for long that IGF-1 stimulated the proliferation of testicular somatic cells such as Sertoli cells and Leydig cells and the production of testosterone [53]. The

effect of IGF-1 on germ cell proliferation was initially studied by a group using cultured rat seminiferous tubules. This study reported that IGF-1 was more potent than IGF-2 in stimulating spermatogonial but not meiotic DNA synthesis [54]. Another study showed that IGF-1 also stimulated the differentiation of type A spermatogonia using the mouse testicular fragments [55]. IGF-1 was also reported to protect rat germ cells from apoptosis induced by testicular torsion [56]. These early studies provide valuable clues for the roles of IGF-1R signaling in spermatogenesis. However, detailed mechanistic studies could not be conducted until the appearance of the in vitro culture system of the mSSCs. In this study, by using cultured mSSCs, we show that IGF-1R signaling promotes the proliferation of cultured mouse spermatogonia, including stem cells and differentiating spermatogonia. It also protects spermatogonia from apoptosis. Our results are consistent with these previous studies and extend them in illustrating some mechanisms of the effect of IGF-1R signaling as discussed next.

Growth factors primarily promote G₀/G₁ and G₁/S transitions [57]. EGF, FGF, and PDGF mainly stimulate quiescent cells to enter G₁, and are termed “competence factors,” whereas IGF-1 promotes progression through G₁ to S phases and was deemed a G₁-progression factor. Sell et al. first reported that IGF-1R signaling had an effect on all stages of the cell cycle by comparing the distributions of the *Igf-1r* KO and wild-type MEFs in each cell cycle stage [58].

A second study reported that IGF-1 had a critical role in G₂ but not G₁ progression of estradiol-induced mitotic cycle of the uterine cells [25]. Moreover, overexpression of the IGF-1R inhibiting protein mGrb10 α caused a delay in the S and G₂ phases of the MEFs [24], and the inhibition of the receptor tyrosine kinase activity of IGF-1R by PPP resulted in the accumulation of multiple myeloma cells in the G₂/M phases [28]. However, IGF-1 was also reported to accelerate the cell cycle of the embryonic cerebral cortex by decreased G₁ phase length based on results using Igf-1 transgenic mice [59], and it also stimulated G₁-phase progression of human intestinal smooth muscle cells [60]. Therefore, the effect of IGF-1R signaling on cell cycle progression seems to be cell type dependent. In this study, for the first time, we report that among all studied growth factors, only IGF-1R signaling has a prominent effect on the G₂/M progression of the cell cycle in mSSCs.

Growth factors activate multiple signaling pathways in target cells. The PI3K-AKT and MAPK1/2 pathways were first reported to be activated by GDNF in mSSCs by Lee et al. [61]. PI3K-specific inhibitor LY294002 prevents mSSCs from self-renewal, while conditional activation of the myristoylated form of Akt-Mer (myr-Akt-Mer) by 4-hydroxy-tamoxifen maintains mSSC in vitro propagation for at least 5 months in the absence of GDNF. Interestingly, the MEK-specific inhibitor PD98059 does not interfere with the growth of mSSCs in the presence of GDNF, FGF2, and EGF. Another study by Lee et al. shows that Ras proteins exert their functions upstream of PI3K, and expression of a constitutively active H-RasV12 protein in mSSCs sustains the long-term growth of cultured mSSCs in the absence of both GDNF and FGF2. The combination of GDNF, FGF2, and EGF induces the expression of cyclin D1, D2, and E, which exert functions at G₁/S phases of the cycle, and the expression of cyclin D2 and E together also maintains mSSC growth in the absence of these growth factors [62]. Pathways activated by IGF-1R signaling include kinase signaling, G-protein signaling, β -arrestin signaling, and so on [63]. In this study, we found that the anti-apoptotic and the G₂/M promoting effects of IGF-1R signaling on mSSCs could be explained by the phosphorylation of AKT. However, the addition of IGF-1 back to serum and growth factor-starved mSSCs was unable to upregulate the phosphorylation of ERK1/2 in mSSCs. That GDNF and FGF2 but not IGF-1 activated the phosphorylation of ERK1/2 partially explains why IGF-1 alone could not support the proliferation and survival of cultured mSSCs.

A decade has passed since the Shinohara and Brinster laboratories reported the establishment of the long-term culture system of mSSCs. However, we still know little about the mechanisms of SSC self-renewal and differentiation. Some factors/signaling pathways beneficiary to the in vitro proliferation of mSSCs have been recognized, but only GDNF and FGF2 signaling are believed to be essential. Here, we report that IGF-1R signaling is the third essential factor for mSSC in vitro propagation. Since IGF-1, IGF-2, and insulin are ligands for IGF-1R, we are not sure which one plays a more pivotal role in IGF-1R signaling. The observations that IGF-1 was expressed by both feeder MEFs and mSSCs and that an excess amount of insulin was unable to rescue the detrimental effect of IGF-1R signaling blockade suggested that IGF-1 was likely, at least partially,

responsible for the activation of IGF-1R signaling. We also observed that withdrawal of exogenous insulin from the culture medium caused mSSC proliferation to stop after two passages (data not included). This adds the essential protein factors of mSSC in in vitro proliferation up to four. However, it is almost certain that more essential factors hide to be identified. This would remain a challenging task with the use of feeder cells, serum, or serum products such as BSA, the elimination of which will greatly contribute to our understanding of mammalian spermatogenesis.

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Author Disclosure Statement

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References

- Oatley JM and RL Brinster. (2008). Regulation of spermatogonial stem cell self-renewal in mammals. *Annu Rev Cell Dev Biol* 24:263–286.
- Nakagawa T, M Sharma, Y Nabeshima, RE Braun and S Yoshida. (2010). Functional hierarchy and reversibility within the murine spermatogenic stem cell compartment. *Science* 328:62–67.
- Spradling A, D Drummond-Barbosa and T Kai. (2001). Stem cells find their niche. *Nature* 414:98–104.
- Scadden DT. (2006). The stem-cell niche as an entity of action. *Nature* 441:1075–1079.
- Yeh JR, X Zhang and MC Nagano. (2011). Wnt5a is a cell-extrinsic factor that supports self-renewal of mouse spermatogonial stem cells. *J Cell Sci* 124:2357–2366.
- Yoshida S, M Sukeno and Y Nabeshima. (2007). A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. *Science* 317:1722–1726.
- Meng X, M Lindahl, ME Hyvonen, M Parvinen, DG de Rooij, MW Hess, A Raatikainen-Ahokas, K Sainio, H Rauvala, et al. (2000). Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science* 287:1489–1493.
- Kanatsu-Shinohara M, N Ogonuki, K Inoue, H Miki, A Ogura, S Toyokuni and T Shinohara. (2003). Long-term proliferation in culture and germline transmission of mouse male germline stem cells. *Biol Reprod* 69:612–616.
- Kubota H, MR Avarbock and RL Brinster. (2004). Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. *Proc Natl Acad Sci U S A* 101:16489–16494.
- Oatley JM, MJ Oatley, MR Avarbock, JW Tobias and RL Brinster. (2009). Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal. *Development* 136:1191–1199.
- He Z, J Jiang, M Kokkinaki and M Dym. (2009). Nodal signaling via an autocrine pathway promotes proliferation of mouse spermatogonial stem/progenitor cells through Smad2/3 and Oct-4 activation. *Stem Cells* 27:2580–2590.

12. Golestaneh N, E Beauchamp, S Fallen, M Kokkinaki, A Uren and M Dym. (2009). Wnt signaling promotes proliferation and stemness regulation of spermatogonial stem/progenitor cells. *Reproduction* 138:151–162.
13. Zhang Y, S Wang, X Wang, S Liao, Y Wu and C Han. (2012). Endogenously produced FGF2 is essential for the survival and proliferation of cultured mouse spermatogonial stem cells. *Cell Res* 22:773–776.
14. He Z, J Jiang, M Kokkinaki, N Golestaneh, MC Hofmann and M Dym. (2008). Gdnf upregulates c-Fos transcription via the Ras/Erk1/2 pathway to promote mouse spermatogonial stem cell proliferation. *Stem Cells* 26:266–278.
15. Le Roith D. (2003). The insulin-like growth factor system. *Exp Diabetes Res* 4:205–212.
16. Annunziata M, R Granata and E Ghigo. (2011). The IGF system. *Acta Diabetol* 48:1–9.
17. Baker J, JP Liu, EJ Robertson and A Efstratiadis. (1993). Role of insulin-like growth factors in embryonic and postnatal growth. *Cell* 75:73–82.
18. Cailleau J, S Vermeire and G Verhoeven. (1990). Independent control of the production of insulin-like growth factor I and its binding protein by cultured testicular cells. *Mol Cell Endocrinol* 69:79–89.
19. Dupont J, A Pierre, P Froment and C Moreau. (2003). The insulin-like growth factor axis in cell cycle progression. *Horm Metab Res* 35:740–750.
20. Schafer KA. (1998). The cell cycle: a review. *Vet Pathol* 35:461–478.
21. Golias CH, A Charalabopoulos and K Charalabopoulos. (2004). Cell proliferation and cell cycle control: a mini review. *Int J Clin Pract* 58:1134–1141.
22. Houtgraaf JH, J Versmissen and WJ van der Giessen. (2006). A concise review of DNA damage checkpoints and repair in mammalian cells. *Cardiovasc Res* 7:165–172.
23. Campisi J and AB Pardee. (1984). Post-transcriptional control of the onset of DNA synthesis by an insulin-like growth factor. *Mol Cell Biol* 4:1807–1814.
24. Morrión A, B Valentinis, M Resnicoff, S Xu and R Basega. (1997). The role of mGrb10 α in insulin-like growth factor I-mediated growth. *J Biol Chem* 272:26382–26387.
25. Adesanya OO, J Zhou, C Samathanam, L Powell-Braxton and CA Bondy. (1999). Insulin-like growth factor I is required for G2 progression in the estradiol-induced mitotic cycle. *Proc Natl Acad Sci U S A* 96:3287–3291.
26. Riedemann J and VM Macaulay. (2006). IGF1R signalling and its inhibition. *Endocr Relat Cancer* 13 (Suppl. 1):S33–S43.
27. Girnita A, L Girnita, F del Prete, A Bartolazzi, O Larsson and M Axelsson. (2004). Cyclolignans as inhibitors of the insulin-like growth factor-I receptor and malignant cell growth. *Cancer Res* 64:236–242.
28. Stromberg T, S Ekman, L Girnita, LY Dimberg, O Larsson, M Axelsson, J Lennartsson, U Hellman, K Carlson, et al. (2006). IGF-1 receptor tyrosine kinase inhibition by the cyclolignan PPP induces G2/M-phase accumulation and apoptosis in multiple myeloma cells. *Blood* 107:669–678.
29. Kanatsu-Shinohara M and T Shinohara. (2010). Germline modification using mouse spermatogonial stem cells. *Methods Enzymol* 477:17–36.
30. Kubota H and RL Brinster. (2008). Culture of rodent spermatogonial stem cells, male germline stem cells of the postnatal animal. *Methods Cell Biol* 86:59–84.
31. Brinster RL and JW Zimmermann. (1994). Spermatogenesis following male germ-cell transplantation. *Proc Natl Acad Sci U S A* 91:11298–11302.
32. Ogawa T, JM Arechaga, MR Avarbock and RL Brinster. (1997). Transplantation of testis germinal cells into mouse seminiferous tubules. *Int J Dev Biol* 41:111–122.
33. Nagano M, MR Avarbock and RL Brinster. (1999). Pattern and kinetics of mouse donor spermatogonial stem cell colonization in recipient testes. *Biol Reprod* 60:1429–1436.
34. Wu Y, S Liao, X Wang, S Wang, M Wang and C Han. (2013). HSF2BP represses BNC1 transcriptional activity by sequestering BNC1 to the cytoplasm. *FEBS Lett* 587:2099–2104.
35. Dou S, YD Yao, XZ Yang, TM Sun, CQ Mao, EW Song and J Wang. (2012). Anti-Her2 single-chain antibody mediated DNMTs-siRNA delivery for targeted breast cancer therapy. *J Control Release* 161:875–883.
36. Yang XY, CC Huang, QM Kan, Y Li, D Liu, XC Zhang, T Sato, S Yamagata and T Yamagata. (2012). Calcium regulates caveolin-1 expression at the transcriptional level. *Biochem Biophys Res Commun* 426:334–341.
37. Tao Q, X Fan, T Li, Y Tang, D Yang and W Le. (2012). Gender segregation in gene expression and vulnerability to oxidative stress induced injury in ventral mesencephalic cultures of dopamine neurons. *J Neurosci Res* 90:167–178.
38. Sun Z, Q Luo, D Ye, W Chen and F Chen. (2012). Role of toll-like receptor 4 on the immune escape of human oral squamous cell carcinoma and resistance of cisplatin-induced apoptosis. *Mol Cancer* 11:33.
39. Tanaka SS, Y Toyooka, R Akasu, Y Katoh-Fukui, Y Nakahara, R Suzuki, M Yokoyama and T Noce. (2000). The mouse homolog of Drosophila Vasa is required for the development of male germ cells. *Genes Dev* 14:841–853.
40. Toyooka Y, N Tsunekawa, Y Takahashi, Y Matsui, M Satoh and T Noce. (2000). Expression and intracellular localization of mouse Vasa-homologue protein during germ cell development. *Mech Dev* 93:139–149.
41. Ruggiu M, R Speed, M Taggart, SJ McKay, F Kilanowski, P Saunders, J Dorin and HJ Cooke. (1997). The mouse Dazl gene encodes a cytoplasmic protein essential for gametogenesis. *Nature* 389:73–77.
42. Buas FW, AL Kirsh, M Sharma, DJ McLean, JL Morris, MD Griswold, DG de Rooij and RE Braun. (2004). Plzf is required in adult male germ cells for stem cell self-renewal. *Nat Genet* 36:647–652.
43. Ebata KT, X Zhang and MC Nagano. (2005). Expression patterns of cell-surface molecules on male germ line stem cells during postnatal mouse development. *Mol Reprod Dev* 72:171–181.
44. Naughton CK, S Jain, AM Strickland, A Gupta and J Milbrandt. (2006). Glial cell-line derived neurotrophic factor-mediated RET signaling regulates spermatogonial stem cell fate. *Biol Reprod* 74:314–321.
45. Sarfstein R and H Werner. (2013). Minireview: nuclear insulin and insulin-like growth factor-I receptors: a novel paradigm in signal transduction. *Endocrinology* 154:1672–1679.
46. Sehat B, A Tofigh, Y Lin, E Trocme, U Liljedahl, J Lagergren and O Larsson. (2010). SUMOylation mediates the nuclear translocation and signaling of the IGF-1 receptor. *Sci Signal* 3:ra10.
47. Sarfstein R, M Pasmanik-Chor, A Yeheskel, L Edry, N Shomron, N Warman, E Wertheimer, S Maor, L Shochat and H Werner. (2012). Insulin-like growth factor-I receptor (IGF-IR) translocates to nucleus and autoregulates IGF-IR gene expression in breast cancer cells. *J Biol Chem* 287:2766–2776.

48. Deng H, Y Lin, M Badin, D Vasilcanu, T Stromberg, H Jernberg-Wiklund, B Sehat and O Larsson. (2011). Over-accumulation of nuclear IGF-1 receptor in tumor cells requires elevated expression of the receptor and the SUMO-conjugating enzyme Ubc9. *Biochem Biophys Res Commun* 404:667–671.
49. Yoshinaga K, S Nishikawa, M Ogawa, S Hayashi, T Kunisada, T Fujimoto and S Nishikawa. (1991). Role of c-kit in mouse spermatogenesis: identification of spermatogonia as a specific site of c-kit expression and function. *Development* 113:689–699.
50. Schrans-Stassen BH, HJ van de Kant, DG de Rooij and AM van Pelt. (1999). Differential expression of c-kit in mouse undifferentiated and differentiating type A spermatogonia. *Endocrinology* 140:5894–5900.
51. Kanatsu-Shinohara M, K Inoue, N Ogonuki, H Morimoto, A Ogura and T Shinohara. (2011). Serum- and feeder-free culture of mouse germline stem cells. *Biol Reprod* 84:97–105.
52. Liu JP, J Baker, AS Perkins, EJ Robertson and A Efstratiadis. (1993). Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). *Cell* 75:59–72.
53. Gnessi L, A Fabbri and G Spera. (1997). Gonadal peptides as mediators of development and functional control of the testis: an integrated system with hormones and local environment. *Endocr Rev* 18:541–609.
54. Soder O, P Bang, A Wahab and M Parvinen. (1992). Insulin-like growth factors selectively stimulate spermatogonial, but not meiotic, deoxyribonucleic acid synthesis during rat spermatogenesis. *Endocrinology* 131:2344–2350.
55. Tajima Y, D Watanabe, U Koshimizu, T Matsuzawa and Y Nishimune. (1995). Insulin-like growth factor-I and transforming growth factor- α stimulate differentiation of type A spermatogonia in organ culture of adult mouse cryptorchid testes. *Int J Androl* 18:8–12.
56. Ozkurkugil C, M Yardimoglu, H Dalcik, S Erdogan and A Gokalp. (2004). Effect of insulin-like growth factor-1 on apoptosis of rat testicular germ cells induced by testicular torsion. *BJU Int* 93:1094–1097.
57. Pardee AB. (1989). G1 events and regulation of cell proliferation. *Science* 246:603–608.
58. Sell C, G Dumenil, C Deveaud, M Miura, D Coppola, T DeAngelis, R Rubin, A Efstratiadis and R Baserga. (1994). Effect of a null mutation of the insulin-like growth factor I receptor gene on growth and transformation of mouse embryo fibroblasts. *Mol Cell Biol* 14:3604–3612.
59. Hodge RD, AJ D'Ercole and JR O'Kusky. (2004). Insulin-like growth factor-I accelerates the cell cycle by decreasing G1 phase length and increases cell cycle reentry in the embryonic cerebral cortex. *J Neurosci* 24:10201–10210.
60. Kuemmerle JF, H Zhou and JG Bowers. (2004). IGF-I stimulates human intestinal smooth muscle cell growth by regulation of G1 phase cell cycle proteins. *Am J Physiol Gastrointest Liver Physiol* 286:G412–G419.
61. Lee J, M Kanatsu-Shinohara, K Inoue, N Ogonuki, H Miki, S Toyokuni, T Kimura, T Nakano, A Ogura and T Shinohara. (2007). Akt mediates self-renewal division of mouse spermatogonial stem cells. *Development* 134:1853–1859.
62. Lee J, M Kanatsu-Shinohara, H Morimoto, Y Kazuki, S Takashima, M Oshimura, S Toyokuni and T Shinohara. (2009). Genetic reconstruction of mouse spermatogonial stem cell self-renewal in vitro by Ras-cyclin D2 activation. *Cell Stem Cell* 5:76–86.
63. Girmata L, C Worrall, S Takahashi, S Seregard and A Girmata. (2014). Something old, something new and something borrowed: emerging paradigm of insulin-like growth factor type 1 receptor (IGF-1R) signaling regulation. *Cell Mol Life Sci* 71:2403–2427.

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