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Tumor Suppressor PTEN Regulates Negatively Sertoli Cell Proliferation, Testis Size, and Sperm Production *In Vivo*

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The IGFs are the major intratesticular factors regulating immature Sertoli cell proliferation and are, therefore, critical to establish the magnitude of sperm production. However, the intratesticular source of IGF production and the downstream signaling pathway mediating IGF-dependent Sertoli cell proliferation remain unclear. Single-cell RNA sequencing on mouse embryonic testis revealed a robust expression of *Igf1* and *Igf2* in interstitial steroidogenic progenitors, suggesting that IGFs exert paracrine actions on immature Sertoli cells. To elucidate the intracellular signaling mechanism that underlies the proliferative effects of IGFs on immature Sertoli cells, we have generated mice with Sertoli cell-specific deletion of the *Pten* gene, a negative regulator of the phosphatidylinositol-3 kinase (PI3K)/AKT pathway, alone or together with the insulin receptor (*Insr*) and the IGF1 receptor (*Igf1r*). Although ablation of *Pten* appears dispensable for Sertoli cell proliferation and spermatogenesis, inactivation of *Pten* in the absence of *Insr* and *Igf1r* rescued the Sertoli cell proliferation rate during late fetal development, testis size, and sperm production. Overall, these findings suggest that IGFs secreted by interstitial progenitor cells act in a paracrine fashion to promote the proliferation of immature Sertoli cells through the IGF/PTEN/PI3K pathway. (***Endocrinology*** **160: 387–398, 2019)**

In adulthood, the main functions of the testes are sperm production and testosterone secretion. Sertoli cells (SCs) are the only somatic constituents of the seminiferous epithelium. These supporting cells are essential for developing germ cells to sustain spermatogenesis by providing them with a unique microenvironment, physical support, and appropriate nutriments, hormones, and growth factors (1). Because each SC can support only a limited number of developing germ cells, the total number of SCs in adulthood will ultimately determine the testis size and daily sperm production (2, 3). The factors regulating SC proliferation and, ultimately, their adult number are critical to establish the magnitude of sperm production. In rodents, SCs proliferate during fetal life, in the postnatal/neonatal period, and just before puberty. After sexual maturation, these cells are then considered mitotically inactive (2, 4). The proliferation of SCs is finely regulated by a combination of hormonal and paracrine/autocrine factors, which regulate the rate and extent of proliferation. In particular, FSH and growth factors of the insulin family, the IGFs, are the most important regulators of SC proliferation (3, 5–8). Other factors and cytokines, including thyroid hormones and activin A, have also been demonstrated to contribute to SC proliferation, although to a lesser extent (9–16).

The insulin/IGF family of growth factors composed of insulin, IGF1, and IGF2 are the major intratesticular factors regulating SC number, testis size, and sperm output in mammals. Recent *in vivo* evidence have indicated that

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Abbreviations: ANV, absolute nuclear volume; EdU, 5-ethynyl-2′-deoxyuridine; IGF1R, IGF1 receptor; INSR, insulin receptor; PI3K, phosphatidylinositol-3 kinase; PTEN, phosphatase and tensin homolog; SC, Sertoli cell.

the insulin/IGF receptors insulin receptor (INSR) and IGF1 receptor (IGF1R) play a major role in regulating immature SC proliferation, maturation, and, ultimately, the total pool of adult SCs (8). The absence of both *Insr* and *Igf1r* in SCs during testis development led to a reduced proliferation rate of immature SCs during the late fetal and early neonatal testicular period, resulting in a 75% reduction in testis size and daily sperm production. In addition, *in vivo* analyses revealed that FSH requires the insulin/IGF signaling pathways to mediate its proliferative effects on immature SCs (8).

However, the precise mechanism explaining how the insulin/IGF signaling pathway promotes SC proliferation has remained unclear. IGF1 and IGF2 act mainly through INSR and IGF1R and activates two major signaling pathways: the ERK/MAPK pathway and the phosphatidylinositol-3 kinase (PI3K)/AKT pathway, both of which are important mediators of growth, proliferation, and cell survival (17, 18). Several lines of evidence have suggested that many of the actions of IGF1 on SCs are dependent on the PI3K/AKT signaling pathway [a review has been reported by Escott *et al.* (19)]. However, the effect of the PI3K/phosphatase and tensin homolog (PTEN) pathway in regulating SC proliferation, testis size, and spermatogenesis has not been determined *in vivo*. As a negative regulator of the PI3K/AKT pathway, the PTEN plays a pivotal role in the regulation of IGF signaling. PTEN acts as a phosphoinositide phosphatase to dephosphorylate phosphatidylinositol(3,4,5) triphosphate to the diphosphate(4,5), thus reducing activation of AKT.

The present study was performed to (i) investigate the testicular source of IGFs and the potential role of PTEN in SCs and (ii) to determine whether the proliferative effects of IGFs on immature SCs is mediated by the PI3K/PTEN signaling pathway. We sought to address these questions using single-cell RNA sequencing to evaluate the expression of IGFs in fetal testis cells and *in vivo* by deleting *Pten* alone or in combination with *Insr* and *Igf1r*, specifically in SCs, and measuring the SC proliferation rates and testis size.

Materials and Methods

Animals

All animal work was conducted according to the ethical guidelines of the Direction Générale de la Santé of the Canton de Genève (authorization identifications, 1061-3840-0, GE/63/16, and GE/57/18). $Insr^{flox}$ (20), $Igf1r^{flox}$ (21), $Pten^{flox}$ (22), *Amh:Cre* (23), and *Sox9:EGFP* (24) mouse strains were described previously. The mice were maintained on a mixed genetic background, and all comparisons were performed between mutant and control littermates. Routine genotyping of the mice was performed using PCR, as previously described (8).

Single-cell capture and cDNA libraries and sequencing

E16.5 testes from wild-type mice were enzymatically dissociated for 10 minutes at 37°C using trypsin-EDTA 0.05% (Gibco, Fisher Scientific, Hampton, NH). After centrifugation and counting, 3000 to 7000 single cells were loaded on a 10× Chromium instrument (10× Genomics, Pleasanton, CA). Single-cell RNA-sequencing libraries were prepared using the Chromium Single Cell 3′, version 2, reagent kit (10× Genomics), according to the manufacturer's protocol. Each stage was performed in two independent replicates. Library quantification was performed using the Qubit fluorometric assay with the double-strand DNA high-sensitivity assay kit (Invitrogen, Carlsbad, CA). The library quality assessment was performed using an Agilent Bioanalyzer 2100 with a high sensitivity DNA chip (Agilent Technologies, Santa Clara, CA). The libraries were diluted, pooled, and sequenced using an Illumina HiSeq4000 using paired-end 26 × 98 bp as the sequencing mode. The libraries were sequenced at a targeted depth of 100,000 to 150,000 reads per cell. The libraries of the two replicates were sequenced on two different runs.

Data availability

FastQ files of the 9478 single-cell RNA sequencing of mouse testis at E16.5 are available on the Gene Expression Omnibus repository (no. GSE123119).

Bioinformatics analysis

The computations were performed at the Vital-IT Centre (available at: http://www.vital-it.ch) for high-performance computing of the SIB Swiss Institute of Bioinformatics. Data were analyzed using R, version 3.5.0 (R Foundation, Vienna, Austria). Highly variable genes were detected using the Seurat FindVariableGenes function (with binning_method = "equal_width" parameter). Principal component analysis was performed on these genes, and cell clustering was performed using the modularity-based community detection clustering algorithm implemented in Seurat function FindClusters (with resolution = 0.1 parameter) on the five first principal components. t-SNE was computed using the fit-SNE algorithm, with 2000 iterations on the five first principal components.

Histological and immunological analyses

Testes were collected, fixed in 4% paraformaldehyde or Bouin fixative, serially dehydrated, embedded in paraffin, and sectioned using a microtome at 5 μm thickness. The Bouin-fixed sections were examined histologically via hematoxylin and eosin staining. Immunofluorescence analyses were performed as described previously (25). Fluorescent images were taken using an Axio Scope.A1 microscope fitted with an AxiocamMRc camera (Carl Zeiss, Oberkochen, Germany) and processed using Zen software (Carl Zeiss).

Proliferation assay

Pregnant mice carrying E17.5 embryos and P5 males were intraperitoneally injected with 1000 μg and 300 μg of 5-ethynyl-2′-deoxyuridine (EdU; Invitrogen), respectively, and the testes were harvested 2 hours afterward. The paraformaldehyde-fixed testis sections were double stained for GATA4 (1:100; sc-9053; Santa Cruz Biotechnology, Dallas, TX) and EdU (Click-iT EdU cell proliferation assay; Invitrogen). A minimum of three different mice

per genotype and a minimum of 50 SCs per mouse were analyzed by blinded observers. For each mouse, three to five sections distant from one another by ≥50 μm were selected. Proliferating SCs are expressed as a percentage of the total SCs and normalized on the control littermates.

Sperm count

Cauda epididymis and vas deferens were manually dissociated in 1 mL of M2 media and incubated for 10 minutes at 37°C to release the sperm cells. Next, 20 μL of a 1:40 dilution of the sperm suspension was loaded in a 100-μm-deep counting chamber (Leja Products, Nieuw-Vennep, Netherlands) and submitted to computer-assisted sperm analysis (CEROS II; Hamilton Thorn Research Inc., Beverly, MA). In each assay, ≥300 sperm cells were analyzed. The number of sperm present in the initial 1-mL suspension was calculated using the computer-assisted sperm analysis system (10^6 sperm cells/mL).

SC count using flow cytometry

Control and mutant testes homozygous for *Sox9-EGFP* were dissected, minced into small pieces, and incubated in DMEM supplemented with collagenase (1 mg/mL C0130; Sigma-Aldrich, St. Louis, MO), hyaluronidase (2 mg/mL H3506; Sigma-Aldrich), and DnaseI (0.8 mg/mL dN25; Sigma-Aldrich) at 37°C for 20 minutes with gentle agitation. The cells were centrifuged for 5 minutes at 1000 rpm, resuspended in trypsin-EDTA 0.05% supplemented with DnaseI (0.8 mg/mL), and incubated for 10 minutes at 37°C with gentle agitation. After centrifugation, the cells were resuspended in 1 mL of PBS without calcium and magnesium and filtered through a 70-μm cell strainer. A 1:10 dilution of the cell suspension was processed with a BD Accuri C6, and the collected data were analyzed with FlowJo software. In brief, the gating strategy consisted of debris exclusion (SSC vs FCS) and dead cell exclusion doublet exclusion (ultraviolet-A vs ultraviolet-H). Finally, green fluorescent protein-negative and green fluorescent protein-positive cell fractions were gated using the FL1 channel.

Determination of testicular cell composition

For histological analysis, Bouin-fixed, paraffin-embedded tissue sections were stained with hematoxylin and eosin using standard protocols. Testicular cell composition was estimated using standard stereological techniques involving point counting of cell nuclei to determine the nuclear volume per testis of SCs and different maturational stages of germ cells, as previously described (26). In brief, cross-sections of testes from control, SC-*Pten*, SC-*Insr*;*Igf1r*, and SC-*Pten*;*Insr*;*Igf1r* mutant mice at P60 were examined using 63× objective fitted to a Zeiss AxioScope A1 and a 121-point eyepiece graticule (Leica Microsystems, Wetzlar, Germany). Applying a systematic sampling pattern from a random starting point, 32 microscopic fields (3872 points) were counted for each animal. Points falling over SC or germ cell nuclei, seminiferous epithelium, interstitium, and seminiferous tubule lumen were scored and expressed as relative (proportion) volume per testis. The values for the percentage of nuclear volume were converted to absolute nuclear volumes (ANVs) per testis by reference to the testis volume (= weight) because shrinkage was minimal.

Statistical analysis

Data were analyzed using the Student *t* test or one-way ANOVA, followed by the Bonferroni post hoc test, and differences with $P < 0.05$ were considered statistically significant. Data are presented as the mean ± SEM.

Results

Expression profile of the IGF family of growth factors and their receptors in the developing testis

We examined the expression profiles of genes coding for members of the family of IGFs, their receptors, and the insulin receptor substrate *Irs1* and *Irs2* in 9478 cells isolated from mouse developing testis at 16.5 dpc using single-cell RNA sequencing (27) (Fig. 1). This particular stage was selected because it corresponds to the period when insulin/IGF signaling has been shown to mediate SC proliferation in the developing testis (8). We classified the different cell populations present in the E16.5 testis using principal component analysis on highly variable genes and a modularity-based community detection clustering algorithm with a low-resolution parameter (28) on the five first principal components (27). We obtained five cell clusters (clusters C1 to C5) (27). Expression enrichment of known markers genes (27) allowed us to assign the identity of the cell clusters to interstitial steroidogenic progenitor cells (C1; n = 6362 cells), SCs (C2; n = 1932 cells), fetal Leydig cells (C3; n = 521 cells), primordial germ cells (C4; n = 483 cells), and endothelial cells (C5; n = 180 cells). We found that transcripts coding for the growth factors *Igf1* were present at high levels in interstitial steroidogenic progenitor cells specifically (Fig. 1). *Igf2* was expressed in both interstitial steroidogenic progenitors and endothelial cells. In contrast, transcripts for *Insr* and *Igf1r* were present at low or medium levels in all cell types, suggesting low, but ubiquitous, expression in testicular cells. The gene coding for the effector protein *Irs2* was expressed in most cell type, although at higher levels in immature SCs, consistent with its role in SC proliferation (29). Overall, the robust expression of *Igf1* and *Igf2* in interstitial steroidogenic progenitors and the presence of transcripts coding for *Insr*, *Igf1r*, and the common effector *Irs2* in immature SCs suggest that IGFs secreted mostly by interstitial progenitor cells promote SC proliferation through paracrine actions.

Pten deletion rescues testis size and sperm production in the absence of *Insr* and *Igf1r*

To investigate whether PTEN acts as a negative regulator of insulin/IGF signaling in immature SCs, we specifically deleted *Pten* (22) alone or combined with *Insr* (20) and *Igf1r* (21) in SCs using an *Amh:Cre* transgene (23). For simplicity, SC-specific deletion of *Pten* (*Pten*$^{fx/fx}$;*Amh:Cre*), *Insr* (*Insr*$^{fx/fx}$;*Amh:Cre*), and *Igf1r* (*Igf1r*$^{fx/fx}$;*Amh:Cre*) have been abbreviated as SC-*Pten*, SC-*Insr*, and

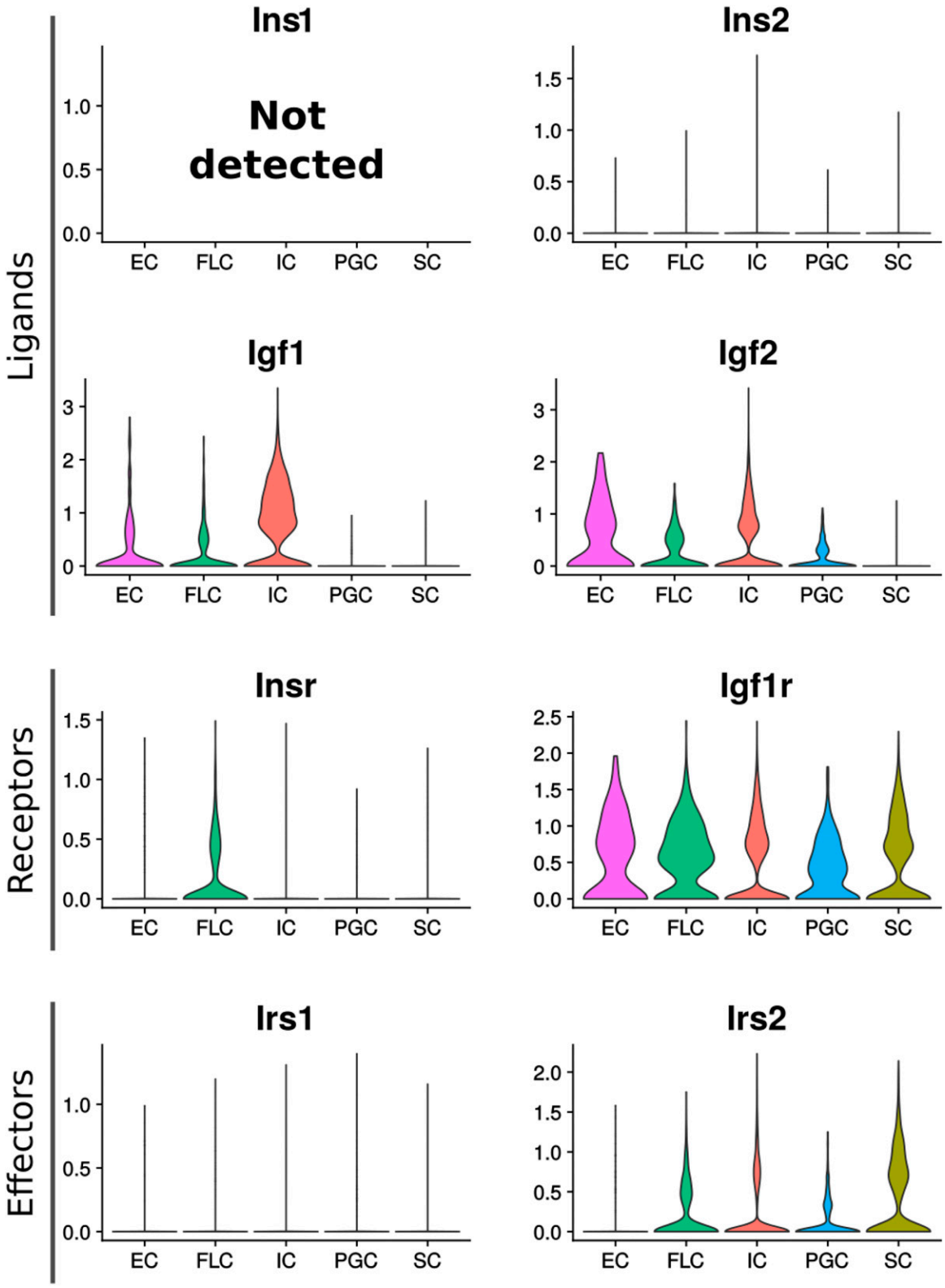

Figure 1. Single cell gene expression levels of IGFs, their receptors and IRS effector proteins in developing testes. The width of the "violin" indicates the frequency at that expression level. Expression scale presented as log-normalized unique molecular identifier (UMI) counts obtained by dividing each gene expression measurement in each cell by the total number of UMI in the cell, multiplying by a scale factor of 10,000 and log-transforming the result. EC, endothelial cell; FLC, fetal Leydig cell; IC, interstitial progenitor cell; PGC, primordial germ cell.

SC-*Igf1r*, respectively. Double (*Insr*$^{fx/fx}$;*Igf1r*$^{fx/fx}$;*Amh:Cre*) or triple (*Pten*$^{fx/fx}$;*Insr*$^{fx/fx}$;*Igf1r*$^{fx/fx}$;*Amh:Cre*) deletions have been referred to as SC-*Insr;Igf1r* and SC-*Pten;Insr;Igf1r*, respectively. Amh-driven Cre recombinase has been reported to efficiently delete floxed alleles specifically in SCs from E14.5 onward (8, 23, 30).

As expected, the adult testes of mice lacking both *Insr* and *Igf1r* in SCs displayed a weight reduction of 70% compared with those of their control littermates (Fig. 2A–2D). However, ablation of one or two *Pten* alleles partially or completely rescued testicular size in the absence of *Insr* and *Igf1r*. Similar results were obtained with epididymal sperm concentrations (Fig. 2E). Although the sperm count was decreased by 83% in SC-*Insr;Igf1r* mutants compared with those in control mice, it was partially or completely rescued in mice lacking one or two functional *Pten* alleles, respectively. Histological analysis of double (SC-*Insr;Igf1r*) and triple (SC-*Pten:Insr;Igf1r*) mutant testes revealed no gross histological alterations of the seminiferous epithelium, with the presence of germ cells at all stages of spermatogenesis and spermiogenesis (data not shown). Taken together, these results have demonstrated that the massive reductions in testis size and sperm output observed in SC-*Insr;Igf1r* mutant animals can be rescued by the ablation of *Pten*, a negative regulator of insulin/IGF signaling in immature SCs.

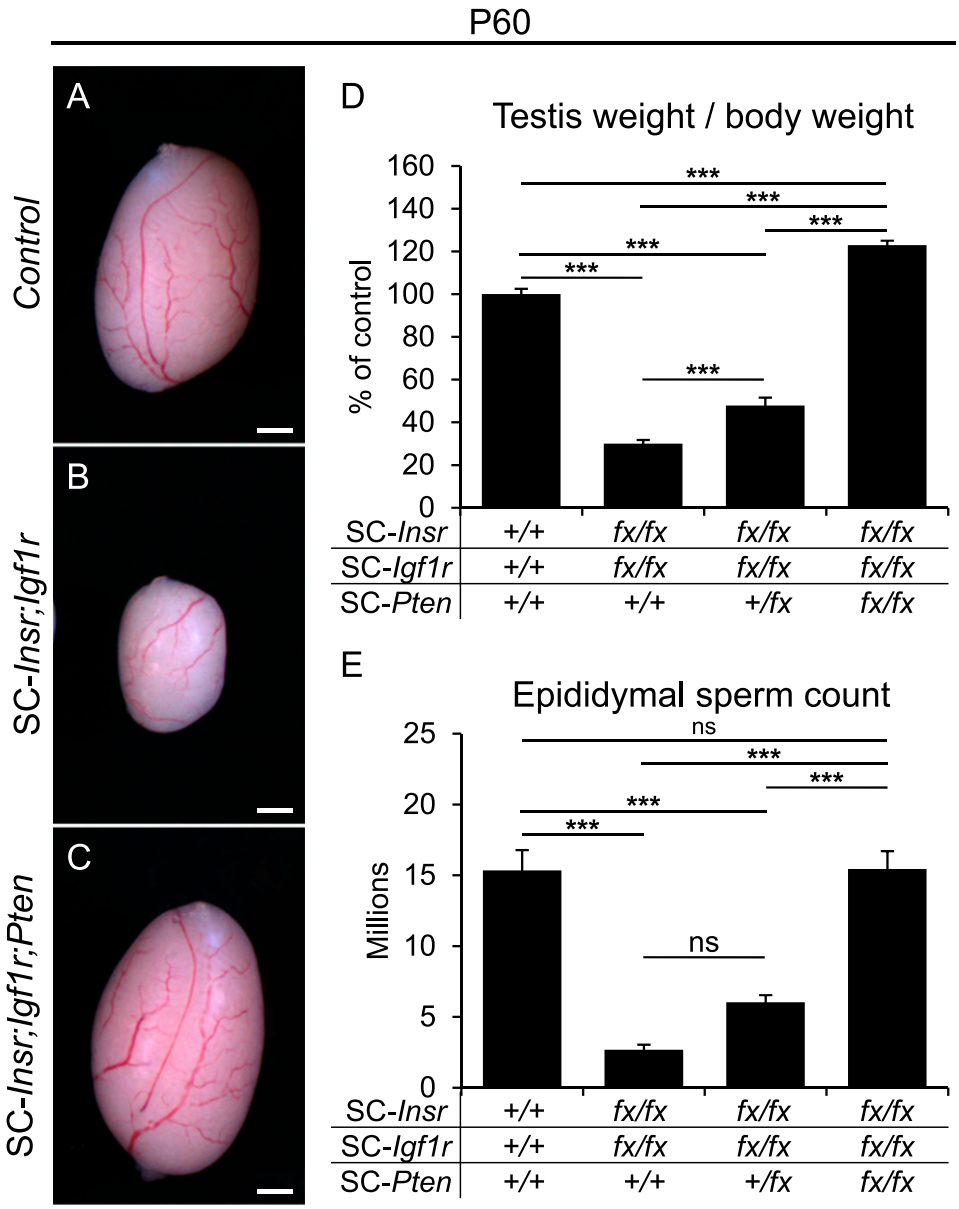

Figure 2. *Pten* deletion rescues testis size and sperm production in the absence of *Insr* and *Igf1r*. Photomicrographs of (A) control, (B) SC-*Insr;Igf1r*, and (C) SC-*Insr;Igf1r;Pten* testes at P60. (D) Testis size was reduced by 70% and 52% in SC-*Insr;Igf1r* and SC-*Insr;Igf1r;Pten*$^{+/flox}$ mice, respectively, compared with controls. In contrast, SC-*Insr;Igf1r;Pten*$^{flox/flox}$ mice display a 21% increase in testis size. Animals used: n = 48 for control, n = 25 for SC-*Insr;Igf1r*, n = 18 for SC-*Insr;Igf1r;Pten*$^{+/flox}$, and n = 25 for SC-*Insr;Igf1r;Pten*$^{flox/flox}$. (E) Quantification of epididymal sperm count revealed an 83% and a 61% decrease in SC-*Insr;Igf1r* (n = 12) and SC-*Insr;Igf1r;Pten*$^{+/flox}$ (n = 14) mice, respectively, compared with controls (n = 15). In contrast, the sperm count in SC-*Insr;Igf1r;Pten*$^{flox/flox}$ (n = 10) mice was unchanged. ***$P < 0.001$ (one-way ANOVA, followed by Bonferroni post hoc test). (A–C) Scale bar, 1 mm. ns, not significant.

Pten deletion rescues SC proliferation and number in the absence of *Insr* and *Igf1r*

The massive reduction in testis size and sperm output observed in SC-*Insr;Igf1r* mice is a consequence of a reduced number of SCs, itself resulting from a decrease in the proliferation rate of immature SCs during the late fetal and early neonatal testicular period (8). We took advantage of the *Sox9;EGFP* knock-in allele to compare testis weight and SC numbers in control and SC-*Insr;Igf1r* and SC-*Pten;Insr;Igf1r* mutant testes at P15. This postnatal stage was selected, because SC proliferation has ceased and the number of SCs has reached its final number. Similar to what has been observed at P60, testis size is reduced by 75% in SC-*Insr;Igf1r* mice but is similar to that of controls in SC-*Pten;Insr;Igf1r* triple mutant testes (Fig. 3A–3D). The same pattern was observed for the number of SCs (although not statistically significant between double and triple mutant testes) confirming that the variation in testis size and sperm output in double or triple mutant animals is directly proportional to the number of SCs (Fig. 3E).

IGF signaling has been shown to promote SC proliferation during late fetal and early neonatal testicular period (8). The absence of IGF signaling led to a reduction in SC proliferation of 55% and 32% at E17.5 and P5, respectively, in SC-*Insr;Igf1r* mice (Fig. 3F). When *Pten* was ablated in the absence of insulin/IGF signaling, the SC proliferation rate returned to normal levels at E17.5 but remained reduced by 35% at P5. These findings indicate that PTEN plays a pivotal role in regulating negatively IGF signaling and SC proliferation during late fetal development. It also explains why the SC number, testis weight, and sperm output remained unaffected in SC-*Pten;Insr;Igf1r* triple mutant mice.

Dispensable role of PTEN in mouse spermatogenesis

Several studies have demonstrated the importance of PTEN and the PI3K/AKT signaling pathway in mouse spermatogenesis, especially in primordial germ cells (31–33). Despite these findings, a role for PTEN in adult SCs and spermatogenesis has never been explored in detail. We found a slight, but statistically significant, increase in testicular weight in SC-*Pten* mice compared with controls at both P15 and P60, although this did not correlate with an increase in SC number and epididymal sperm counts (Fig. 4A–4G). Histological examination of adult testes from SC-*Pten* and control mice revealed that SC-*Pten* mice had grossly normal testicular histological features, with no major changes to seminiferous tubule organization, although in some rare instances, we found seminiferous tubules with spermatogenic defects (Fig. 4H–4J).

A detailed stereological examination of adult testes from control, SC-*Pten*, SC-*Insr;Igf1r*, and SC-*Pten;Insr;Igf1r* was performed to determine whether the testicular cell composition was affected. More precisely, we used standard stereological techniques to determine the ANV per testis of SCs, spermatogonia, spermatocytes, round spermatids, and elongated spermatids for each mutant lines (see "Materials and Methods" for more details; Table 1).

Concerning SC-*Insr;Igf1r* double mutant mice, we observed a dramatic reduction of the ANV of SCs (threefold reduction) and total germ cell (four-time reduction). In the population of germ cells, all the main types showed reduced ANV, including spermatogonia (threefold reduction), spermatocytes (fourfold reduction), round spermatids (fourfold reduction), and elongated spermatids (sixfold reduction). These results suggest that spermatogenesis is negatively affected by the absence of IGF signaling in SCs. In contrast, no major alteration in testicular cell composition was observed in both SC-*Pten* and SC-*Pten;Insr;Igf1r* mutant mice. A comparison of the data between double and triple mutant mice clearly demonstrated substantial recovery of spermatogenesis, because the values of ANV of the SC, total germ cells, spermatogonia, spermatocytes, and round and elongated spermatids were several times greater in the triple mutant mice. Overall, these findings suggest that spermatogenesis was not affected in mice lacking *Pten* either alone (SC-*Pten*) or combined with *Insr* and *Igf1r* (SC-*Pten;Insr;Igf1r*). Elimination of the antagonistic action of PTEN on the PI3K signaling pathway is probably able to compensate for the absence of functional INSR and IGF1R in mutant SCs.

Discussion

IGFs are the main factors promoting SC development and proliferation. In the present study, we have demonstrated that the major source of IGFs is the interstitial steroidogenic progenitor cells and that PTEN, a major negative regulator of the PI3K/AKT signaling pathway, acts as a critical regulator of IGF-dependent SC proliferation, thus affecting both testis size and daily sperm output. Although conditional deletion of *Pten* alone in immature SCs does not affect the SC number or spermatogenesis, inactivation of *Pten* in the absence of *Insr* and *Igf1r* rescued the SC proliferation rate during late fetal development. These findings suggest that the IGF/PTEN/PI3K pathway mediates the proliferative actions of IGFs in immature SCs.

Major source of IGFs in developing testes are interstitial steroidogenic progenitor cells

Our single-cell RNA sequence analysis of 9478 cells from E16.5 testes revealed that the major testicular

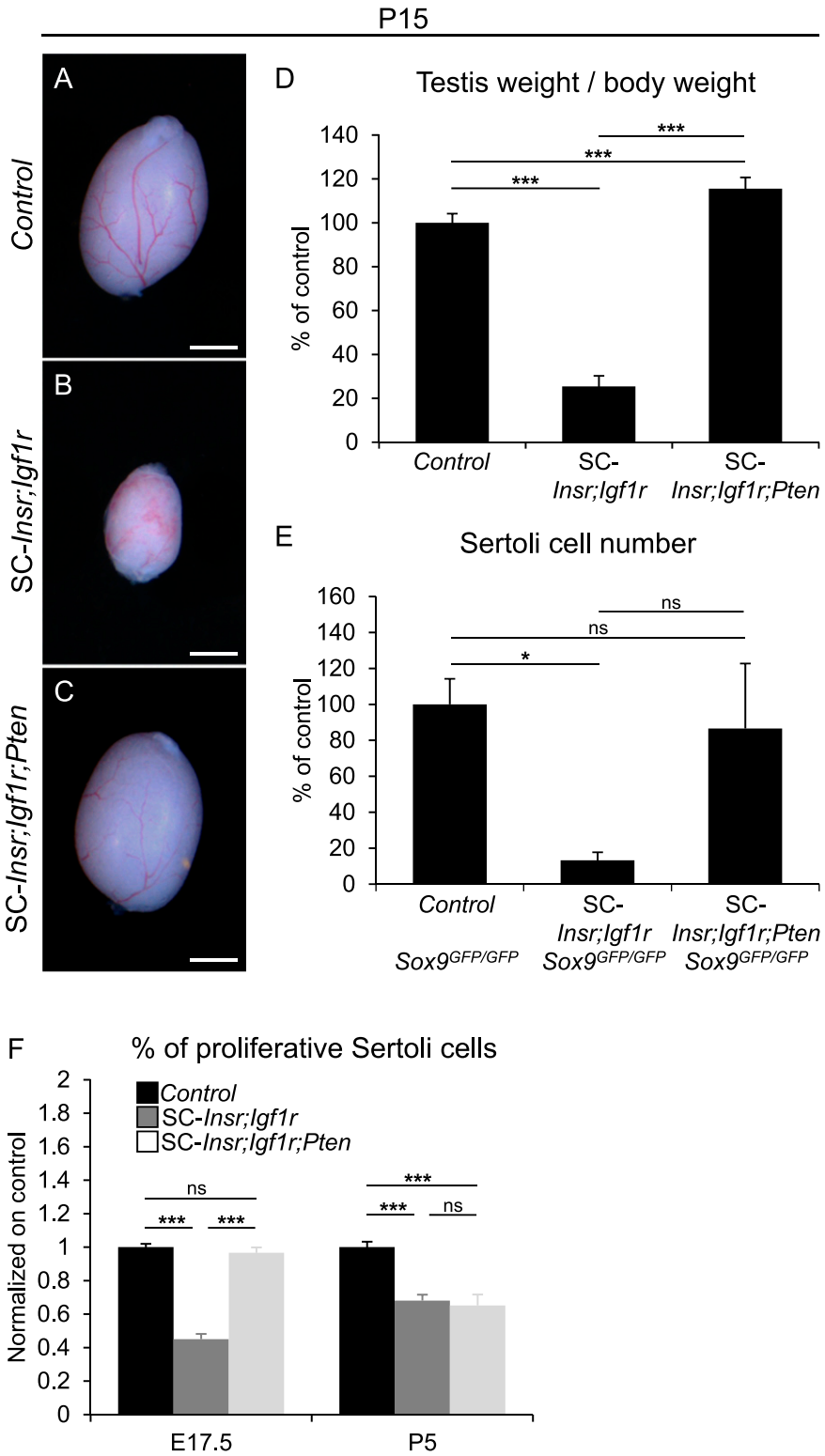

Figure 3. *Pten* deletion rescues SC proliferation and total number in the absence of *Insr* and *Igf1r.* Photomicrographs of (A) control, (B) SC-*Insr;Igf1r*, and (C) SC-*Insr;Igf1r;Pten* testes at P15. (D) Testis size was reduced by 75% in SC-*Insr;Igf1r* compared with controls. In contrast, SC-*Insr;Igf1r;Pten*$^{flox/flox}$ mice displayed a 16% increase in testis size. Animals used: n = 28 for control, n = 8 for SC-*Insr;Igf1r*, and n = 12 for SC-*Insr;Igf1r;Pten*$^{flox/flox}$. (E) SC number was reduced by 87% in SC-*Insr;Igf1r* (n = 3) mice compared with controls (n = 13). In contrast, the proportion was unchanged in SC-*Insr;Igf1r;Pten*$^{flox/flox}$ (n = 6) mice. (F) Proliferating SCs were decreased by 55% and 32% at E17.5 (n = 4) and P5 (n = 3), respectively, in SC-*Insr;Igf1r* mice compared with controls (E17.5, n = 6; P5, n = 7). In SC-*Insr;Igf1r;Pten*$^{flox/flox}$ mice, proliferating SCs were not affected at E17.5 (n = 3) but displayed a 35% decrease at P5 (n = 3). $*P < 0.05$; $***P < 0.001$. (A–C) Scale bar, 1 mm. ns, nonsignificant.

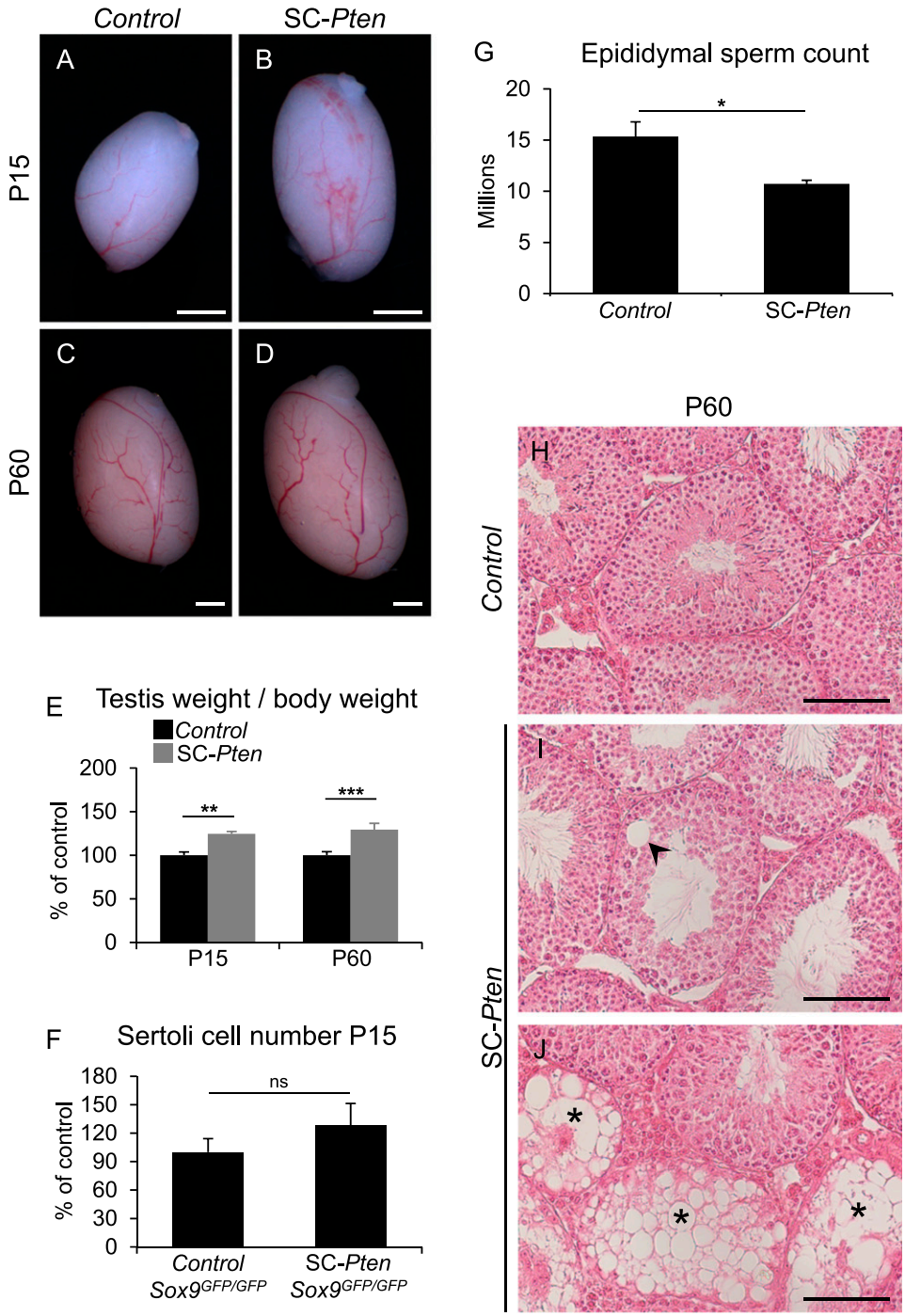

Figure 4. Male fertility defect in the absence of *Pten*. Photomicrographs of control (A,C) and SC-*Pten* (B,D) testes at P15 (A,B) and P60 (C,D). (E) Testis size was increased by 12% and 14% in SC-*Pten* compared with controls at P15 and P60, respectively. Animals used at P15: n = 28 for control, n = 10 for SC-*Pten*. Animals used at P60: n = 48 for control, n = 21 for SC-*Pten*. (F) SC number at P15 was similar between controls (n = 13) and SC-*Pten* (n = 7) mice. (G) Quantification of epididymal sperm count reveals a 30% decrease in SC-*Pten* (n = 8) compared with controls (n = 15). Histological hematoxylin and eosin-stained sections of (H) control and (I,J) SC-*Pten* testes showing vacuole (black arrowhead) and SC-only tubules (asterisk) in mutant animals at P60. $*P < 0.05$; $**P < 0.01$; $*** P < 0.001$. Scale bars, (A–C) 1 mm and (H–J) 100 μm. ns, nonsignificant.

source of IGFs are the interstitial progenitor cells, although *Insr* and *Igf1r* are expressed at low levels in all cell types. It suggests strongly that IGF1 and IGF2 secreted from these somatic interstitial progenitors regulate, in a paracrine action, SC proliferation and, thus, testis size and sperm output. However, it is currently impossible to test *in vivo* whether the interstitial source of IGFs mediates immature SC proliferation simply because the transgenic lines required for such experiments are either are missing or inefficient. First, no Cre line is available with specific expression in interstitial steroidogenic cell progenitors. The main reason is that the characterization

Table 1. SC and Germ Cell Composition of the Testes From Control, *SC-Pten*, *SC-Insr;Igf1r*, and *SC-Pten;Insr;Igf1r* Mutant Mice

	Average Nuclear Volume (mm^3/testis)					
Genotype	Sertoli	Total Germ Cell	Spermatogonia	Spermatocyte	Round Spermatid	Elongate Spermatid
Control (n = 11)	1.22 ± 0.08	13.86 ± 0.64	0.77 ± 0.09	6.72 ± 0.38	4.49 ± 0.35	1.88 ± 0.16
SC-Pten (n = 7)	1.30 ± 0.16	13.74 ± 1.10	0.62 ± 0.07	7.03 ± 0.63	4.26 ± 0.38	1.83 ± 0.32
SC-*Insr;Igf1r* (n = 3)	0.37 ± 0.06[a]	3.23 ± 0.21[a]	0.28 ± 0.02[a]	1.60 ± 0.12[a]	1.05 ± 0.04[a]	0.31 ± 0.05[a]
SC-Insr;Igf1r;Pten (n = 5)	1.56 ± 0.06[b,c]	16.15 ± 1.33[c]	0.76 ± 0.17[d]	7.54 ± 0.32[c]	5.36 ± 0.90[e]	2.48 ± 0.20[c]

Data presented as the mean ± SEM.

[a] $P < 0.001$ (compared with control value).

[b] $P < 0.01$ (compared with control value).

[c] $P < 0.001$ (comparison of *SC-Insr-Igf1r* with *SC-Insr-Igf1r-Pten* mutant mice).

[d] $P < 0.05$ (comparison of *SC-Insr-Igf1r* with *SC-Insr-Igf1r-Pten* mutant mice).

[e] $P < 0.01$ (comparison of *SC-Insr-Igf1r* with *SC-Insr-Igf1r-Pten* mutant mice).

of the steroidogenic progenitors is very recent (34), without enough time to generate and test the penetrance and specificity of such a Cre transgenic line. Second, we found that Cre-mediated LoxP deletion with the available *Igf1* floxed line (35) was inefficient. In an attempt to test the testicular function of IGF1 and IGF2, we specifically deleted these two genes either in germ cells or in SCs. Although deletion of the two *Igf2* alleles occurred successfully using either the *Amh:Cre* line (23) or the *Ddx4:Cre*^{ERT2} line (36), *Igf1* Cre-mediated lox deletion was partial with ~30% of *Igf1* lox alleles deleted (data not shown).

Immature SC proliferation is mediated by the IGF/PTEN/PI3K pathway

On ligand binding, INSR and IGF1R activate multiple downstream signaling cascades, with the two most prominent PI3K/AKT and RAS/MAPK (37, 38). The PI3K/AKT pathway is fundamental for regulation of multiple cellular processes including cell proliferation differentiation and apoptosis (39, 40). The PI3K/AKT signaling pathway is inhibited by PTEN, a lipid and protein phosphatase that hydrolyzes PIP3 to PIP2 and prevents PIP3 membrane recruitment and stimulation of AKT (41). Therefore, loss of PTEN phosphatase activity leads to activating cell survival, growth, and proliferation (42, 43). It is no surprise that PTEN loss promotes tumorigenesis in multiple organs, in both humans and animal models (44). However, elucidating the *in vivo* role of PTEN during testis development using conventional deletion of *Pten* is impossible because homozygous *Pten*-null mice die during early embryogenesis (45–47). Conditional deletion of *Pten* in primordial germ cells caused testicular teratomas resulting from enhanced proliferation (32). In contrast, we have shown in the present study that specific deletion of *Pten* in developing SCs does not affect testis development and spermatogenesis. However, this absence of phenotype in SC-*Pten* mice might simply reflect that the PI3K signaling pathway is already fully active owing to IGF and/or FSH action. We found that specific deletion of *Pten* in SCs that also lack insulin/IGF signaling (SC-*Pten;Insr;Igf1r* triple mutant mice) rescued IGF-mediated SC proliferation. It indicates also that INSR and IGF1R mediate SC proliferation through the PTEN/PI3K pathway.

PTEN/PI3K downstream effectors mediating SC proliferation remain unclear

Although it is clear that PTEN and PI3K play critical roles in promoting SC proliferation with IGF stimulation, it remains unclear which of the downstream signal transduction pathways, including the PI3K/AKT and MAPK pathways, are involved. Multiple lines of evidence suggest that SC proliferation is mediated by the PI3K/AKT pathway in response to either FSH or IGFs (19, 48–50). AKT plays a critical role in cell survival, growth, proliferation, differentiation, and metabolism [for a review see Manning and Cantley (51)]. Activated AKT can stimulate proliferation through phosphorylation of multiple downstream targets impinging on cell cycle regulation. It includes the cell cycle inhibitors p21 and p27 (52, 53), the glycogen synthase kinases 3α and 3β) (54), the tuberous sclerosis complex 2 (55), the proline-rich AKT substrate of 40 kDa (56), the mTOR complex 1 (57), and transcription factors such as forkhead transcription family members (58). Extensive research on the wiring of signal transduction pathways has revealed that AKT controls cell proliferation through multiple complementary downstream pathways, none of them operating in isolation. To date, the downstream effectors mediating the PI3K/AKT signal pathways remain unknown.

It also remains possible that IGFs mediate part of their mitotic action through the MAPK pathway. In P5 rat SCs, FSH-dependent proliferation correlates with enhanced phosphorylation of ERK1/2, thus increasing cyclin D1 (59). PI3K can associate directly with RAS to activate it and, thereby, the MAPK pathway. PTEN is also a negative regulator of ERK1/2 (MAPK) through mechanisms that are not very clear (60). Deletion of PTEN in several organs and cells has also been associated with basal overactivation of ERK1/2. Thus, it remains possible that the deletion of PTEN restores the defects observed with the deletion of the INSR/IGFR, also by overactivating MAPK, and not just AKT. Although technically difficult, it would be interesting to compare the phosphoproteome of E16.5 SCs in control and SC-*Insr;Igf1r* double mutant mice to identify which AKT and/or ERK effectors are phosphorylated exclusively in control conditions.

Disentangling FSH and IGF action in mediating SC proliferation

Both IGFs and FSH exert important mitotic action on immature SCs. These hormones do not act independently; instead they interact with each other to regulate SC proliferation (8, 61). For example, insulin/IGF signaling has been shown to mediate *in vivo* FSH proliferative action on immature SCs (8). However, the specific mechanism by which IGFs and FSH interact to promote SC proliferation require further elucidation. One hypothesis has suggested that the interaction between IGFs and FSH is the result of converging signaling pathways. Both IGFs and FSH mediate their proliferative activity on immature SCs through the activation of a common PI3K/AKT pathway (61, 62). Alternatively, the interaction between FSH and IGF actions could be indirect, with FSH increasing the secretion of endogenous IGF1 and decreasing IGFBP3, thus promoting PI3K/AKT activation (62). However, further investigation is needed to clarify how FSH and IGF1 cooperate to promote SC proliferation.

In conclusion, it is now clear that IGFs promote SC proliferation via the activation of IGF/IRS2/PI3K signaling (27, 63) and that PTEN acts as a critical negative regulator of IGF-dependent SC proliferation. Our ability to understand the complex circuitry of PTEN/PI3K signaling and how it controls SC proliferation through multiple complementary downstream pathways remain challenging. The work of piecing it all together and figuring out how it really works lies ahead.

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