Inactivation of the *IGF-I* receptor gene in primary Sertoli cells highlights the autocrine effects of IGF-I

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Abstract

IGF-I regulates pituitary and gonadal functions, and is pivotal for sexual development and fertility in mammalian species. To better understand the function of autocrine IGF-I in Sertoli cell physiology, we established a system for Cre-mediated conditional inactivation of the IGF-I receptor (IGF-IR) in cultured Sertoli cells. We show here that loss of IGF-IR decreased the number of viable Sertoli cells as a consequence of diminished Sertoli cell proliferation and increased Sertoli cell death.

Furthermore, the lack of IGF-IR altered the morphology of cultured Sertoli cells and decreased lactate and transferrin secretions. Collectively, our data indicate that autocrine IGF-I contributes significantly to Sertoli cell homeostasis. The described *in vitro* system for loss-of-function analysis of the IGF-IR can be readily transposed to study the role of other intratesticular growth factors involved in spermatogenesis.

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Introduction

Proliferation, differentiation, and apoptosis of male germ cells are finely regulated by pituitary hormones, essentially luteinizing hormone and follicle-stimulating hormone (FSH; McLachlan *et al.* 2002), and by a network of factors originating from both the different somatic cells of the testis (Sertoli cells, Leydig cells, and peritubular cells) and the germ cells. Since most of the factors produced within the testis are also widely expressed in other organs, the attempts to understand their role in spermatogenesis by conventional knockout strategies have been limited. Some mice die shortly after birth (e.g. knockout of insulin-like growth factor-I, IGF-I, Liu *et al.* 1993; transforming growth factor β (TGF- β), Oshima *et al.* 1996; nerve growth factor (NGF), Tessarollo 1998), or they do not exhibit any particular phenotype on male fertility (e.g. knockout of IGF-II, IGF-binding protein-2 to -6 (IGFBP-2 to -6); Chandrasekar *et al.* 2004). Moreover, even if for some of these factors produced by, and acting on, testicular cells, knockout models or spontaneous genetic defects have allowed us to understand their role on the early steps of spermatogenesis (e.g. stem cell factor (SCF),

Besmer *et al.* 1993, Glial cell line-derived neurotrophic factor (GDNF), Meng *et al.* 2000), their action, if any, on later steps of spermatogenesis could not be studied since invalidation of these genes results in an early blockade of the spermatogenic process (GDNF, Meng *et al.* 2000). In addition, ubiquitous factors (such as IGF-I, TGF- β) may be expressed by several cell types in the testis and their role can change during development and spermatogenesis (Kierszenbaum 1994).

Sertoli cells play a crucial role during spermatogenesis (Review: Petersen & Soder 2006). They support germ cells in the seminiferous tubule and provide them with growth factors, binding proteins (like androgen binding protein or transferrin), and energy in form of lactate thereby promoting germ cell growth and differentiation into spermatozoa. The secretion of these factors contributes to a microenvironment, which can regulate the balance between self-renewal of spermatogonia and their differentiation, similarly to what occurs in other stem cell niches (Moore & Lemischka 2006). Thus, some testicular cancers or a reduction in sperm quality and/or quantity could result from Sertoli cell dysfunction.

IGF-I, a pleiotropic cytokine, stimulates metabolism, proliferation, differentiation, and survival of numerous cell

types (Cohick & Clemmons 1993). Circulating IGF-I is mainly produced by the liver, but most other cell types also produce IGF-I which can act in an autocrine/paracrine manner (Humbel 1990). Importantly, IGF-I and its cognate tyrosine kinase receptor (IGF type I receptor, IGF-IR) are expressed in the testis (Hansson *et al.* 1989). IGF-I is produced by the Leydig cells, the Sertoli cells, the peritubular/myoid cells, and the germ cells; and its receptor is found in the germ cells and the somatic testicular cells (Tres *et al.* 1986, Vannelli *et al.* 1988, Cailleau *et al.* 1990). The complexity of the IGF family of proteins (ligands, receptors, high-affinity binding proteins) and their versatile mode of action (endocrine versus local) in combination with the cellular heterogeneity of the testicular tissue complicate the physiological interpretation of experimental data. Several investigations have suggested that IGF-I regulates important testicular functions, including germ cell proliferation and survival, testosterone production by the Leydig cells, and stimulate the activity of the Sertoli cells (Borland *et al.* 1984, Baker *et al.* 1996, Froment *et al.* 2004).

To determine the physiological functions of autocrine IGF-I in Sertoli cells, we disrupted the IGF-IR *in vitro* in both immature and more differentiated Sertoli cells, cultured in media containing minimal amounts of exogenous IGF-I. Then, we studied the consequences of the loss of IGF-IR on proliferation, survival, and specific differentiated functions (lactate and transferrin secretions) of primary Sertoli cells.

Materials and Methods

Mouse Sertoli cell culture

Sertoli cells were obtained from mice with a homozygous loxP-based conditional mutation of the *IGF-IR* gene (IGF-IR^{lox/lox} mice, Holzenberger *et al.* 2000a,b, Desbois-Mouthon *et al.* 2006). Experiments were conducted according to institutional guidelines for the care and use of laboratory animals. Efficiency of Cre-loxP recombination in cultured cells was determined using triplex PCR for the simultaneous detection of intact (floxed) and null (Cre-recombined) alleles (Leneuve *et al.* 2001, see Fig. 3a). Sertoli cell culture and reagents were previously described (Weiss *et al.* 1997, Le Magueresse-Battistoni *et al.* 1998); for each culture, 20–25 male mice from different litters of the same age were pooled. Briefly, Sertoli cells were isolated from 10- or 20-day-old mice by two successive collagenase digestions (Sigma) followed by 0.1% hyaluronidase treatment (Sigma) for 20 min at 33 °C to reduce peritubular cell contamination (Le Magueresse-Battistoni *et al.* 1998). To detect peritubular myoid cells contamination, an alkaline phosphatase (a marker for peritubular myoid cells) staining was performed as previously described (Le Magueresse-Battistoni *et al.* 1998). The percentage of alkaline phosphatase-positive cells was close to 3–5% of the total cell population and contamination of Sertoli cells preparation with germ cells (<10%) where no longer present after a few days in culture. The cells were

seeded in HEPES-buffered F12/Dulbecco's modified Eagle's medium (DMEM) medium (Life Technologies) with 5% fetal calf serum (FCS) at 33 °C in a humidified atmosphere of 5% CO₂ in air. After 12 h, the medium was changed to 1% FCS, and after 48 h, Sertoli cells were trypsinized and subcultured in appropriate plates in the presence of the latter medium with lentivirus referred to as day 0 of infection. We used 96-well plates for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (3–5 × 10³ cells/well), 6-well plates for cell death and protein analysis (5 × 10⁵ cells/well), and chamber slides for 5-bromodeoxyuridine (BrdU) incorporation (2 × 10⁴ cells/chamber; all plates were obtained from Nunc, Naperville, IL, USA). For gene expression analysis, immunostaining, lactate and transferrin immunoassay, Sertoli cells were seeded in bicameral chambers (area 1 cm²; polyester membrane, pores 0.4 µm diameter; Greiner Bio-one, Dutscher, Issy-les-moulineaux, France) at 3 × 10⁵ cells/cm². The medium was changed every 48 h. Cells were stimulated by recombinant human IGF-I (a gift from Dr P Swift, Ciba-Geigy, Saint Aubin, Switzerland) or oFSH (ovine NIH FSH-20, obtained through NIDDK and Dr A F Parlow, lot AFP-7028D).

Production of lentiviral vectors and infections

The vesicular stomatitis virus envelope glycoprotein G-pseudotyped HIV-1-derived vectors (empty lentivirus: mock, or lenti-green fluorescent protein (GFP), lenti-Cre-GFP, lenti-Cre) were generated as described (Negre *et al.* 2000). Lenti-Cre and lenti-Cre-GFP were kindly provided by Prof. A Pfeifer, Ludwig-Maximilians University, Munich, Germany. Sertoli cells were infected with lentivirus in the presence of 6 µg/ml polybrene (Sigma) for 12 h at a multiplicity of infection of 20 per cell.

Reverse transcription-PCR

Total RNA extraction and reverse transcription were performed as described (Froment *et al.* 2003). Primers for cDNA amplification are in Table 1. PCR conditions were 1 min denaturation at 94 °C, 30 s annealing at 58 °C, and 30 s extension at 72 °C using an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany). Control PCR with untranscribed RNA was performed in parallel (data not shown). PCR amplification was stopped after 24–26 cycles before reaching the plateau.

Immunoprecipitation and immunoblotting

Cell lysates were prepared as described (Froment *et al.* 2003). Extracts containing 200 µg protein were incubated with IGF-IR antibody (1:1000 dilution, C20, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 16 h at 4 °C. Immune complexes were precipitated by incubation with protein G agarose for 1 h at 4 °C as described (Dupont *et al.* 2000).

Table 1 Oligonucleotide primer sequences

	Accession no	Sense (5'–3')	Antisense (5'–3')	Product (pb)	Cycles
MRNA					
IGF-I	NM_010512	GCTGGTGGATGCTCTTCAGTT	CTTCTCCTTGCAGCTTCGTTT	270	30
IGF-IR	NM_010513	TTCTTCTATGTCCCGCCAAA	AGCCTCGTTTACCGTCTTGAT	356	30
IRS1	NM_010570	ACTTGAGCTATGACACGGCT	GGTTGGAGCAACTGGATGAA	389	30
IRS2	XM_976196	CTCTGACTATATGAACCTGG	ACCTTCTGGCTTTGGAGGTC	339	30
Cyclin D2	NM_009829	AGCTGTCCCTGATCCGCAAG	GTCAACATCCCGCACGTCTG	350	26
LDHA	BC094019	ACAGTCCACACTGCAAGCTG	TTCCACTGCTCCTTGTCTGC	304	26
Transferrin	BC058218	GGCATCGGACACTAGCATCA	TGCCATCAGGGCAGAGCAAC	390	26
Inhibin	NM_010564	TCAGCCCAGCTGTGGTTCACA	AGCCCAGCTCTTGAAGGAGAT	440	26
18S	BK000964	CGACGACCCATTCGAACGTCT	GCTATTGGAGCATGGAATTACCG	312	24

Immunoprecipitates or protein extracts were subjected to SDS-PAGE under reducing conditions and transferred onto nitrocellulose membrane (Schleicher and Schuell, Ecqueville, France) for 2 h as described in Froment *et al.* (2003).

Antibodies against phosphorylated extracellular signal-regulated kinase (ERK)1/2 (Thr202/Tyr204 pERK), phosphorylated Akt (Ser473 pAkt), Akt, cyclin D2, cleaved caspase-3, phosphorylated Bad (Ser135), phosphorylated glycogen synthase kinase-3 β (GSK-3 β) (Ser9), and GSK-3 β were purchased from Cell Signaling (Beverly, MA, USA). Antibodies against IGF-IR (C20), ERK1/2, p21 (F5), and β -catenin were purchased from Santa Cruz Biotechnology. A sheep polyclonal antibody against p53 (Ab-7) was obtained from Oncogene (Oncogene Science, Boston, MA, USA). Antibodies were used at 1:1000 dilution except anti-p53 (1:3000). Anti-GFP (1:500), anti-Cre (1:1000), and anti-phosphotyrosine (PY20; 1:1000) antibodies were obtained from Roche (Roche Applied Science), Novagen (Novagen, San Diego, CA, USA), and Transduction Laboratories (Lexington, KY, USA) respectively. Vinculin monoclonal antibody (hVIN-1, 1:1000, Sigma) was used for normalization. As secondary antibodies, HRP-linked sheep anti-mouse IgG, donkey anti-rabbit IgG (1:10 000, Amersham Biosciences), or rabbit anti-sheep IgG (1:10 000, Upstate Biotechnology, Mundolsheim, France) were used. The signals were detected by ECL (Amersham Pharmacia Biotech). Signals were quantified using Scion Image software (Scion Corporation, Frederick, MD, USA) and normalized with vinculin or total protein (for phosphorylated protein). Results correspond to the average of three cell lysates per genotype and are expressed as the signal intensity in arbitrary units.

Viability and cell proliferation

Cell viability was estimated by a MTT (Sigma) colorimetric assay based on the conversion of MTT to MTT-formazan product by mitochondrial dehydrogenases from living cells. Four hours prior to the measurement, the medium was replaced by a medium without red phenol and containing 10% MTT solution. Then, an MTT solvent containing isopropanol and 0.1 N HCl was added. The absorbance of the

purple MTT formazan was measured spectrophotometrically using a microplate reader. The amount of MTT formazan produced is proportional to the number of viable cells. Results correspond to the average of three independent experiments each performed in triplicate.

For BrdU incorporation, the cultured cells were labeled for 24 h with 10 μ M BrdU (Sigma) 7 days after infection. The cells were then fixed for 10 min in 4% paraformaldehyde (PAF)/PBS. BrdU-positive cells were identified by indirect immunofluorescence as described (Migliorini *et al.* 2002) and counted in at least 20 different microscopic fields with a minimum of 1000 cells in each condition.

Numbers of dead cells were counted after trypan blue staining.

Immunocytochemistry

For actin, β -catenin, and cleaved caspase-3 immunofluorescence, cells were infected with lentivirus (lenti-Cre, lenti-Cre-GFP, or mock) in bicameral chambers. Seven days after infection, cells were fixed for 10 min in 4% PAF/PBS, washed with PBS, and incubated in 0.1 M glycine/PBS for 15 min. Cells were washed again and then permeabilized for 20 min with 0.15% Triton X-100 (w/v) in PBS containing 1% BSA. Nonspecific binding sites were blocked by incubating in 2% BSA/PBS for 20 min. Cells were then incubated for 60 min with 0.5 μ M fluorescein iso thio cyanate (FITC)-conjugated phalloidin (actin staining; Sigma) or anti- β -catenin or an anti-cleaved caspase-3 antibodies (Santa Cruz Biotechnology; Cell Signaling) and washed extensively in PBS. β -Catenin and cleaved caspase-3 antibodies were revealed with a goat cyanine 3-conjugated antirabbit antibody (1:100, 30 min, Amersham Biosciences), and cells were counterstained with diaminido phenyl indole (DAPI) before mounting.

The size of plated Sertoli cells was determined after actin staining. Using a 100 $\times$ objective lens, two perpendicular sections per cell were measured to estimate the cell surface in at least 100 cells/condition. The intensity of the β -catenin staining signal over a constant area (15 $\times$ 15 pixels) was measured with the Scion Image software (Scion Corporation). A minimum of 50 measurements/condition from ten

different microscopic fields was collected and the mean result expressed in arbitrary units.

p70S6K in vitro assay

P70S6K activity was measured using the Cell Signaling assay (Cell Signaling). Sertoli cell lysate (described under immunoprecipitation and immunoblotting) was immunoprecipitated with an anti-p70S6K antibody (Cell Signaling) and the specific enzyme activity estimated by measuring the phosphorylation of an artificial substrate (AKRRRLSSLRA corresponding to an 11-amino acid fragment of the ribosomal protein S6) via incorporation of γ -³²P-ATP.

Lactate and transferrin assay

Lactate concentration was determined using a commercial spectrophotometric assay (Lactate Pap, Bio Merieux, Marcy-L'Etoile, France) with a detection limit of 0.07 mmol/l.

Murine transferrin concentration was measured by RIA as previously described (Cassia *et al.* 1997). The linear range of the assay was 0.1–50 ng/tube with an intra-assay variation coefficient of 8% for samples within a 20–70% binding. The detection limit was <1 ng/ml. All standards and samples were assayed in triplicate.

Values of lactate and transferrin concentrations are the mean $\pm$ S.E.M. of four independent experiments each performed in triplicate.

Statistical analysis

Data were presented as the mean $\pm$ S.E.M. Paired *t*-test was used to compare treated cells with their corresponding control when *n* $\geq$ 4. When required, values were logarithmically transformed to eliminate heterogeneity of variance and achieve a reasonable assumption of normal distribution (Bland & Altman 1996). If values do not assume Gaussian distribution, a nonparametric paired test was performed. Data obtained from IGF-I- and FSH-stimulated cells were compared by one-way ANOVA followed by the *post hoc* Fisher test. *P* < 0.05 was considered significant.

Results

Efficient deletion of floxed IGF-IR by lentiviral Cre delivery

To study the role of IGF-IR in immature and more differentiated Sertoli cells, we purified cells from the testis of 10-day-old (10 dpp, immature Sertoli cells) and 20-day-old (20 dpp, more differentiated Sertoli cells, noted as mature cells) homozygous IGF-IR^{lox/lox} mice (Holzenberger *et al.* 2000a,b). Indeed, in the mouse, Sertoli cells stop dividing 12 days after birth (Kluin *et al.* 1984).

We showed that in culture, the immature and mature Sertoli cells expressed as expected the major components of

Figure 1 Sertoli cell purification and expression of IGF components. Expression of IGF-I, IGF-IR, and its intracellular substrates IRS-1 and IRS-2 was analyzed by RT-PCR in purified Sertoli cells prepared from 10- and 20-day-old mice. The expected sizes of the products are in Table 1. 18S served as a loading control. Ctrl, negative control.

the *IGF-I* gene family, including the ligand IGF-I, its cognate receptor IGF-IR and the intracellular receptor substrates IRS1 and IRS2 (Fig. 1). The cultured IGF-IR^{lox/lox} Sertoli cells were then infected with lentivirus expressing GFP or empty vector (lenti-GFP or mock, as controls) or Cre-GFP (lenti-Cre-GFP) to trigger the inactivation of the *IGF-IR*

Figure 2 Lentiviral infection of Sertoli cells. Representative microscopic field of immature Sertoli cells infected with lenti-Cre-GFP. Cells were counterstained 5 days after infection with DAPI (right picture).

gene *in vitro*. More than 85% of the Sertoli cells were infected and expressed the GFP, as determined by fluorescence activated cell sorting (FACS) analysis (data not shown) and fluorescence microscopy (Fig. 2). Expression of Cre in Sertoli cells led to efficient excision of exon 3 from the *IGF-IR* gene

as determined by exon 3-specific PCR on Sertoli cell genomic DNA (Fig. 3a). Although loss of exon 3 from *Igf1r* does a priori not change the synthesis of IGF-IR mRNA, we observed a decrease in IGF-IR mRNA steady-state levels at 48 h by RT-PCR analysis (Fig. 3b). The excision of exon 3

Figure 3 Conditional inactivation of the *IGF-IR* gene after infection by lenti-Cre virus. (a) Left panel: Schematic representation of the 5' end of the mouse *IGF-IR* gene (not to scale) with exon 3 flanked by loxP sites (triangles). Upon Cre expression, exon 3 was excised. Primers (5'–3'): (a) CCATGGGTGTAAATGTTAATGGC, (b) ATCTTGGAGTGGTGGGTCTGTTTC, and (c) ATGAATGCTGGT-GAGGGTGTCTT were used to simultaneously identify floxed, excised, and wild-type (WT) *IGF-IR* alleles. Right panel: Efficient excision after infection was confirmed by PCR amplification of genomic DNA from *IGF-IR*^{+/lox} or *IGF-IR*^{lox/lox} immature Sertoli cells. NI, non-infected; GFP, lenti-GFP virus infected; Cre-GFP, lenti-Cre-GFP virus infected; ctrl, negative control. (b) Semi-quantitative RT-PCR analysis of immature Sertoli cells infected with lenti-Cre-GFP virus showing a decrease of IGF-IR mRNA from 48 h post-infection onwards. 18S served as a loading control. (c) Western blot analysis of immature Sertoli cells infected with lenti-Cre-GFP virus. The IGF-IR protein was undetectable from day 7 after infection onwards. Vinculin served as a loading control. (d) IGF-I-induced tyrosine phosphorylation of IGF-IR was determined by western blot. Immature Sertoli cells were infected with lenti-GFP virus (GFP), lenti-Cre-GFP virus (Cre-GFP), or remained uninfected (NI). After 7 days, cells were stimulated with 100 ng/ml IGF-I for 15 min and cell lysates immunoprecipitated with IGF-IR antibody. Immunoprecipitates (IP IGF-IR) were immunoblotted with an antibody against phosphotyrosine (PY20) to reveal the phosphorylation of the β -subunit of IGF-IR. Supernatants (cell lysates depleted in IGF-IR, IP supernatant) were immunoblotted using antibodies against Cre and GFP to confirm the lentiviral infection. Vinculin served as a loading control. (e) Analysis of ERK1/2 and Akt phosphorylation by western blot analysis. Immature Sertoli cells were infected with lenti-GFP virus (GFP) or lenti-Cre-GFP virus (Cre). Seven days later, cells were stimulated with 100 ng/ml IGF-I for 15 min or 100 ng/ml FSH for 60 min. Cell lysates were immunoblotted with antibodies against phospho-ERK1/2, ERK1/2, phospho-Akt, and Akt. Vinculin served as a loading control. Representative results of three independent experiments ($n=3$).

creates a premature stop in exon 4 (Holzenberger *et al.* 2000a) that destabilizes mature transcripts and could explain the decreased transcript abundance. Importantly, the IGF-IR protein levels decreased progressively upon Cre expression and IGF-IR were undetectable from 7 days post-infection onwards (Fig. 3c). Hence the following experiments were performed beyond 7 days after infection. Tyrosine phosphorylation of the IGF-IR β -subunit in response to IGF-I (100 ng/ml for 15 min) was strong in controls (non-infected and lenti-GFP-infected Sertoli cells). In contrast, tyrosine phosphorylation upon IGF-I stimulation was dramatically reduced in lenti-Cre-GFP-infected cells (Fig. 3d) consistent with a lack of IGF-IR availability. The capacity of activation by IGF-I (100 ng/ml for 15 min) or FSH (100 ng/ml for 60 min) of the two main signaling pathways downstream of IGF-IR, the PI3K/Akt pathway and the MAPK (ERK1/2) pathway (LeRoith *et al.* 1995), was evaluated. As could be expected (Crepieux *et al.* 2001, Khan *et al.* 2002), IGF-I and FSH stimulation in controls increased phosphorylation of Akt (tested on Ser473) and ERK1/2 (tested on Thr202/Tyr204) (Fig. 3e). Consistently, the lack of IGF-IR in the Cre-infected cultures reduced the IGF-I-induced activation of Akt by $24 \pm 5\%$ (lenti-Cre-GFP versus lenti-GFP, $P=0.012$, $n=3$) and diminished the activation of ERK1/2 by $14 \pm 0.5\%$ ($P=0.003$, $n=3$). Meanwhile, under FSH stimulation, the loss of IGF-IR did not affect Akt and ERK1/2 activation (Fig. 3e). Overall, these results confirm the efficiency and specificity of the method to delete IGF-IR. Thus, in the following experiments, we investigated the impact of IGF-IR depletion on proliferation, cell death, morphology, and secreting activity of immature or differentiated Sertoli cells.

Cell growth was reduced and cell death was increased in the absence of IGF-IR

In the absence and also in the presence of FSH (20 ng/ml), lack of the IGF-IR protein reduced the number of viable Sertoli cells (Fig. 4a) and decreased by 30% the number of immature Sertoli cells taking up BrdU in comparison with control cells (Cre $4.9 \pm 0.7\%$ of BrdU-positive cells vs GFP $7.5 \pm 1.1\%$ of BrdU-positive cells, $P=0.031$, $n=3$; Fig. 4b).

Furthermore, the mRNA and protein levels of cyclin D2, a major G1/S progression factor, appeared to be reduced in the absence of IGF-IR (Fig. 4c; mRNA level, 46% reduction; protein level, 38% reduction). Lastly, both p53 and p21CIP1/Waf1 proteins, p21 is a p53 target gene encoding a cyclin dependent kinase (CDK) inhibitor that acts at the G1/S checkpoint, appeared higher in the absence of IGF-IR (Fig. 4c).

In differentiated Sertoli cells after 11 days of culture, the absence of IGF-IR led to a fourfold increase in cell death (lenti-Cre-GFP: $9.5 \pm 1.5\%$ dead cells vs lenti-GFP: $2.3 \pm 1.2\%$, $P=0.050$, $n=3$, Fig. 4e). Moreover, Sertoli cell death appeared correlated with dephosphorylation of Ser136 of Bad and Ser9 of GSK-3 β (Fig. 4f). In addition, the level of cleaved caspase-3 was increased in cells lacking IGF-IR, as determined by western blot (Fig. 4f) and immunohistochemistry (Fig. 4g). Similar results were observed with immature Sertoli cells (not shown).

Morphology of Sertoli cells was changed in the absence of IGF-IR

In vivo, Sertoli cells maintain close contact with other Sertoli cells and with germ cells via several types of junctions (Goossens & Van Roy 2005). One type of junction, the cadherin/catenin complex, is known to be regulated by IGF-I in colorectal cancer cell lines (Playford *et al.* 2000). Interestingly, we found that on day 11 post-infection, among Sertoli cells devoid of IGF-IR, the intercellular spaces were conspicuously increased (Fig. 5a) and the estimated average Sertoli cell size was reduced (lenti-Cre: $10\,084 \pm 650 \mu\text{m}^2$ compared with mock: $16\,245 \pm 920 \mu\text{m}^2$, $P<0.001$, $n=3$). Moreover, the intensity of the β -catenin staining at the membrane level of Sertoli cells deficient for IGF-IR was lower than in control cells (lenti-Cre: 26.9 ± 0.8 vs mock: 51.7 ± 1.28 arbitrary units, $P<0.001$, $n=3$, Fig. 5b and c).

Sertoli cell differentiated functions were altered in the absence of IGF-IR

To evaluate differentiated functions of mature Sertoli cells, we measured mRNA expression, and/or production, of transferrin, lactate, and inhibin, major products of differentiated Sertoli cells. The mRNA levels encoding for transferrin, lactate

Figure 4 Effect of IGF-IR inactivation on survival and cell growth of Sertoli cells. (a) Immature Sertoli cells were infected with lenti-Cre-GFP (phenotype IGF-IR negative at day 7 post-infection) virus or lenti-GFP (phenotype IGF-IR positive) viruses and cultured with FSH (20 ng/ml) or without for 13 days. The number of viable cells was determined indirectly by MTT assay. Values are the mean $\pm$ S.E.M. of three independent experiments performed in triplicate. (b) Immature Sertoli cells were labeled with BrdU for 24 h, 7 days after infection with lenti-Cre-GFP virus (phenotype IGF-IR negative) and lenti-GFP (phenotype IGF-IR positive), cells were then stained with an anti-BrdU antibody and analyzed by indirect immunofluorescence ($n=3$). (c) Levels of cyclin D2 mRNA and protein in immature Sertoli cells, 7 days after infection with lenti-GFP (phenotype IGF-IR positive) and lenti-Cre-GFP (phenotype IGF-IR negative), as determined by semi-quantitative RT-PCR (left panel, similar results were obtained in two other experiments) and by western blot (right panel, $n=4$). 18S and vinculin served as loading controls. (d) Levels of p53 and p21 proteins in immature Sertoli cells, 7 days after infection with viruses (refer Fig. 4c), as determined by western blot. Vinculin served as a loading control. Similar results were obtained in two other experiments. (e) Percentage of dead mature Sertoli cells in culture on days 5 and 11 after infection with lenti-GFP (GFP) and lenti-Cre-GFP (Cre), as evaluated by trypan blue staining ($n=3$). (f) Levels of the cleaved form of caspase-3, Ser136-phosphorylated Bad, Ser9-phosphorylated GSK-3 β , and total GSK-3 β protein as determined by western blot in mature Sertoli cells infected 7 days before (viruses, refer Fig. 4c). Vinculin serves as a loading control. Similar results were obtained in a second independent experiment. (g) Representative microscopic fields of the cleaved caspase-3 staining (arrows) in mature Sertoli cells infected 7 days before with mock (phenotype IGF-IR positive) or lenti-Cre-GFP (phenotype IGF-IR negative). Cells were counterstained with DAPI. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

dehydrogenase (LDHA, enzyme producing lactate), and inhibin were decreased in Sertoli cells deficient for IGF-IR (transferrin: -64% , $P=0.012$, $n=4$; LDHA: -53% , $P=0.011$, $n=5$; inhibin: -59% , $P=0.027$, $n=5$; Fig. 6a). In addition, the secreted amounts of transferrin and lactate in the apical medium were reduced (Fig. 6b). P70S6Kinase is a key enzyme regulating protein synthesis and cell growth and is stimulated by IGF-I and FSH in Sertoli cells (Lecureuil *et al.* 2005). The lack of IGF-IR in Sertoli cells reduced the p70S6Kinase activity induced by either IGF-I (15 min, 100 ng/ml, $P<0.001$, $n=3$) or FSH (60 min, 100 ng/ml, $P=0.003$, $n=3$; Fig. 6c).

Discussion

The present study aimed to investigate the potential autocrine action of IGF-I on Sertoli cell survival, proliferation, and

differentiation using deletion of IGF-IR. Indeed, we observed expression of IGF-I in Sertoli cells (Fig. 1) and it has been shown previously that IGF-I is released into the medium by cultured Sertoli cells (Skalli *et al.* 1992). The use of the Cre-lentivirus technology gives us the opportunity to study growth factor action at different times of cell maturation. Presently, *in vivo* inactivation of genes in Sertoli cells can only be realized at an early stage of gonad differentiation (14.5 day post coitum, anti-Müllerian hormone (AMH)-Cre mouse line, Lecureuil *et al.* 2002). In contrast, our methodological approach allowed to delete the IGF-IR in immature (10 dpp) and more differentiated (20 dpp) Sertoli cells. Under our culture conditions, mouse Sertoli cells are not able to survive in the absence of FCS. However, we reduced the amount of FCS in the culture medium to 1%, thereby keeping the concentration of serum-derived IGF-I (and other growth

Figure 5 Effect of IGF-IR inactivation on Sertoli cell morphology. (a) Representative microscopic fields of cultured mature Sertoli cells in bicameral chambers 11 days after lentiviral infection (Micrographs 1 and 2, mock infected, and 3 and 4, lenti-Cre infected). Actin was stained with FITC-conjugated phalloidin. Note that the intercellular spaces between the Sertoli cells deleted for the IGF-IR (3,4) was increased (arrows) as compared with control cells (1,2). (b) Localization of β -catenin immunofluorescence (arrows) in mature Sertoli cells, 11 days after infection with mock (phenotype IGF-IR positive) and lenti-Cre (phenotype IGF-IR negative). (c) Intensity of β -catenin staining at the membrane levels was measured in three independent experiments as indicated under Materials and Methods. *** $P<0.001$.

factors) to minimum levels. A 1% serum provides no more than 0.7 ng IGF-I per ml of medium (Kurtz *et al.* 1985), which is five- to ten-fold lower than the ED50 of IGF-I on Sertoli cells as reported in several studies (Jaillard *et al.* 1987, Rappaport & Smith 1995, 1996). Under our experimental conditions, we found that more than 85% of Sertoli cells were infected by lentivirus. In the infected cultures, we observed an almost complete loss of exon 3 from the *IGF-IR* gene and a near complete disappearance of IGF-IR protein from the Sertoli cell population. This in turn led to a decreased activation of the ERK1/2 and Akt pathways, immediately downstream of IGF-IR.

An important consequence of the absence of IGF-IR was a decrease in the number of viable cells. This could be due to a reduction in cell proliferation and/or to an increase in cell death. We observed fewer cells in the S phase, a decrease in cyclin D2 expression, stabilization/activation of p53, and upregulation of p21, suggesting a cell cycle arrest in G1/S. In addition, the number of dead Sertoli cells was increased as well as the level of activated caspase-3, and this was associated with dephosphorylation of Bad. Previous *in vitro* studies have shown that Sertoli cell proliferation can be enhanced by addition of IGF-I to the culture medium (Borland *et al.* 1984). Moreover, in other cell types, IGF-I has been shown to

Figure 6 Lack of IGF-IR altered the activity of mature Sertoli cells. (a) Semi-quantitative RT-PCR analysis of transferrin, LDHA, and inhibin mRNAs in mature Sertoli cells. Cells were infected 11 days before with viruses as in Fig. 3c. 18S served as a loading control ($n=4-5$). (b) Transferrin and lactate secretion by mature Sertoli cells. Cells were cultured for 9 days after virus infection as in Fig. 3c. Transferrin and lactate concentrations in the apical medium were measured every 48 h after complete change of the medium. Values are the mean $\pm$ S.E.M. of four independent experiments performed in triplicate. (c) p70S6K activity in mature Sertoli cell extracts. Sertoli cells were infected, as in Fig. 3c, 7 days before stimulation with 100 ng/ml IGF-I for 15 min, or with 100 ng/ml FSH for 60 min ($n=3$). (a-c) * $P<0.05$.

activate survival signals (reviewed by Kurmasheva & Houghton 2006) and negatively regulates proapoptotic genes such as Bad (Datta *et al.* 1997) and GSK-3 β (Kuemmerle 2005). Disruption of the *p21* gene in transgenic mice leads to an increase in the number of Sertoli cells, daily sperm production, and testis weight (Holsberger *et al.* 2005). Moreover, stabilization of p53 in transgenic mice (Francoz *et al.* 2006) induces apoptosis by activating the caspase-3 pathway in several cell types, including neurons, muscle cells, and hematopoietic cells. The decrease of the size of Sertoli cells associated with an increase of the intercellular spaces between the Sertoli cells could be the consequences of apoptosis induction. Taken together, these results support the view that the local (autocrine) IGF-I production is involved in both proliferation and survival of Sertoli cells.

The activity of p70S6Kinase, the secretion of lactate and transferrin, and inhibin expression were decreased in Sertoli cells deficient for the *IGF-IR* gene. These results are in accordance with studies showing that addition of IGF-I or micromolar concentrations of insulin to Sertoli cell cultures increases incorporation of [3 H]leucine (Borland *et al.* 1984), lactate and transferrin productions (Borland *et al.* 1984, Oonk *et al.* 1989), and glucose transport by the Sertoli cells (Mita *et al.* 1985). Several *in vitro* studies have shown FSH and IGF-I

take part in the regulation of Sertoli cell activity (Khan *et al.* 2002). The effects of FSH and IGF-I when added together to cultured cells may be additive (Khan *et al.* 2002) or antagonistic (Rappaport & Smith 1996). In the present study, deletion of the IGF-IR in Sertoli cells clearly decreased the mitotic action of FSH as well as the response of p70S6K activity to FSH (Lecureuil *et al.* 2005). These results reinforce the view that the local production of endogenous IGF-I is indeed physiologically relevant and could interfere with another major signal in the regulation of Sertoli cell function as summarized schematically in Fig. 7.

As the main role of Sertoli cells is to support the spermatogenic process by nursing the germ cells and given the consequences of the IGF-IR knockout on several functions of the differentiated Sertoli cell *in vitro*, we would expect that the isolated lack of IGF-IR in Sertoli cells *in vivo* engendered significant consequences for sperm production. Whereas IGF-I-deficient mice (Baker *et al.* 1996) and Snell and Ames dwarf mice (Chubb & Nolan 1985) are sterile, several models of dwarf mice, with low or undetectable IGF-I circulating level such as GH receptor-deficient mice (Laron syndrome; Chandrasekar *et al.* 1999) or mice overexpressing hIGFBP-1 (Froment *et al.* 2004), despite exhibiting small testis and/or alteration of testosterone secretion, reduction of

Figure 7 Schematic summary of the cellular and molecular consequences of IGF-IR deletion in Sertoli cells.

sperm production, and in some cases increased germ cell apoptosis are still able to reproduce, although with reduced efficiency. This suggests that the local secretion of IGF-I can be involved in testis maturation. However, none of the above studies have determined if the action of local IGF-I is exerted directly on the germ cells, and/or indirectly via the Sertoli cells or the Leydig cells. IGF-I is known to stimulate testosterone, which is involved in germ cell survival and maturation (Lin *et al.* 1986). Inactivation of the androgen receptor only in Sertoli cell *in vivo* (De Gendt *et al.* 2004) increases also apoptosis of germ cells and induces alterations of meiosis. The present results extend these observations by demonstrating for the first time an autocrine action of IGF-I on Sertoli cells and therefore independent of testosterone produced by the Leydig cells.

Surprisingly, a preliminary study has shown no alteration of sperm production and fertility after conditional deletion of IGF-IR in Sertoli cells *in vivo* (AMH-Cre; IGF-IR^{lox/lox} mice, P Monget, F Guillou, M Holzenberger, unpublished results). Since this deletion of IGF-IR occurred very early during fetal life, it might be hypothesized that early loss of IGF-I signaling was compensated by other signals present in the developing testis. Hence, in order to clarify the potential implication of auto/paracrine IGF-I in Sertoli-germ cell interaction, co-culture of germ cells (Weiss *et al.* 1997, Vigier *et al.* 2004) together with wild-type or IGF-IR deleted Sertoli cells should be helpful.

The methodological approach used in the present work has provided additional support to the view that IGF-I is an autocrine factor involved in both survival and maintenance of the differentiated functions of Sertoli cells at different stages of differentiation. This approach could be used in the future to analyze the consequences of loss-of-function of other factors, produced in the testis, on Sertoli cell physiology.

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