

Thyroid Hormone Limits Postnatal Sertoli Cell Proliferation In Vivo by Activation of Its Alpha1 Isoform Receptor (TRalpha1) Present in These Cells and by Regulation of Cdk4/JunD/c-myc mRNA Levels in Mice 1

Authors: Fumel, Betty, Guerquin, Marie-Justine, Livera, Gabriel, Staub, Christophe, Magistrini, Michèle, et al.

Source: *Biology of Reproduction*, 87(1)

Published By: Society for the Study of Reproduction

URL: <https://doi.org/10.1095/biolreprod.111.098418>

The BioOne Digital Library (<https://bioone.org/>) provides worldwide distribution for more than 580 journals and eBooks from BioOne's community of over 150 nonprofit societies, research institutions, and university presses in the biological, ecological, and environmental sciences. The BioOne Digital Library encompasses the flagship aggregation BioOne Complete (<https://bioone.org/subscribe>), the BioOne Complete Archive (<https://bioone.org/archive>), and the BioOne eBooks program offerings ESA eBook Collection (<https://bioone.org/esa-ebooks>) and CSIRO Publishing BioSelect Collection (<https://bioone.org/csiro-ebooks>).

Your use of this PDF, the BioOne Digital Library, and all posted and associated content indicates your acceptance of BioOne's Terms of Use, available at www.bioone.org/terms-of-use.

Usage of BioOne Digital Library content is strictly limited to personal, educational, and non-commercial use. Commercial inquiries or rights and permissions requests should be directed to the individual publisher as copyright holder.

BioOne is an innovative nonprofit that sees sustainable scholarly publishing as an inherently collaborative enterprise connecting authors, nonprofit publishers, academic institutions, research libraries, and research funders in the common goal of maximizing access to critical research.

Thyroid Hormone Limits Postnatal Sertoli Cell Proliferation In Vivo by Activation of Its Alpha1 Isoform Receptor (TRalpha1) Present in These Cells and by Regulation of Cdk4/JunD/c-myc mRNA Levels in Mice¹

Betty Fumel,^{3,4,5,6} Marie-Justine Guerquin,⁷ Gabriel Livera,⁷ Christophe Staub,⁸ Michèle Magistrini,^{3,4,5,6} Christophe Gauthier,^{3,4,5,6} Frédéric Flamant,⁹ Florian Guillou,^{3,4,5,6} and Sophie Fouchécourt^{2,3,4,5,6}

³INRA, UMR85 Physiologie de la Reproduction et des Comportements, Nouzilly, France

⁴CNRS, UMR6175 Physiologie de la Reproduction et des Comportements, Nouzilly, France

⁵Université François Rabelais de Tours, Tours, France

⁶IFCE, Nouzilly, France

⁷INSERM, U967, CEA/DSV/iRCM/SCSR/LDG, Université Paris Diderot, Sorbonne Paris Cité, Fontenay-Aux-Roses, France

⁸INRA UE 1297—Unité Expérimentale de Physiologie Animale de l'Orfrasière, Nouzilly, France

⁹IGFL (Institut de Génomique Fonctionnelle de Lyon) UMR 5665 ENSL/CNRS INRALA 913, IFR128 Biosciences Lyon Gerland, Lyon, France

ABSTRACT

Hypo- and hyperthyroidism alter testicular functions in the young. Among T3 receptors, TRalpha1 is ubiquitous, and its previously described knockout leads to an increase in testis weight and sperm production. We tested, for the first time, the hypothesis that TRalpha1-dependent regulation of Sertoli cell (SC) proliferation was directly regulated by TRalpha1 present in these cells. Thus, after crossing with the AMH-Cre line, we generated and analyzed a new line that expressed a dominant-negative TRalpha1 isoform (TRalpha^{AM1}) in SCs only. So-called TRalpha^{AM1}-SC (TRalpha^{AM1/+} Cre⁺) mice exhibited similar phenotypic features to the knockout line: heavier testicular weight and higher sperm reserve, in comparison with their adequate controls (TRalpha^{AM1/+} Cre⁻). SC density increased significantly as a result of a higher proliferative index at ages Postnatal Day (P) 0 and P3. When explants of control testes were cultured (at age P3), a significant decrease in the proliferation of SCs was observed in response to an excess of T3. This response was not observed in the TRalpha^{AM1}-SC and knockout lines. Finally, when TRalpha^{AM1} is present in SCs, the phenotype observed is similar to that of the knockout line. This study demonstrates that T3 limits postnatal SC proliferation by activation of TRalpha1 present in these cells. Moreover, quantitative RT-PCR provided evidence that regulation of the Cdk4/JunD/c-myc pathway was involved in this negative control.

Sertoli cells, T3, TRα1

¹Supported by INSERM (grant "Programme National de Recherche en Reproduction et Endocrinologie"; 2006–2007) and the ANR (Agence Nationale pour la Recherche; grant "Biodiversité, évolution des écosystèmes, écosystèmes productifs, agronomie"; 2011–2013). B.F. is a fellow of the Région Centre, France.

²Correspondence: Sophie Fouchécourt, PRC INRA F-37380, Nouzilly, France. E-mail: sfouchecourt@tours.inra.fr

Received: 15 December 2011.

First decision: 9 January 2012.

Accepted: 19 April 2012.

© 2012 by the Society for the Study of Reproduction, Inc.

This is an Open Access article, freely available through *Biology of Reproduction's* Authors' Choice option.

eISSN: 1529-7268 <http://www.biolreprod.org>

ISSN: 0006-3363

INTRODUCTION

Sertoli cells (SCs) are essential in spermatogenesis, as they play a variety of important roles in seminiferous tubules. They create cytoplasmic crypts around germ cells and provide nutrients and growth factors. They also play an immunological role through the creation of specialized tight junctions and the establishment of the blood-testis barrier. It is well established that the number of SCs determine the efficiency of spermatogenesis, as one SC can only support a limited number of germ cells [1].

It is generally believed that SCs proliferate at birth, and the proliferation rate then declines rapidly during the neonatal period before ceasing completely at around the age of 15 days in rodents [2, 3] when SCs enter into differentiation concomitantly with the establishment of the blood-testis barrier [4]. However, Ahmed et al. [5] showed that postpubertal SCs cannot be regarded as terminally differentiated cells, as they possess a residual proliferation capacity in vivo. Moreover, proliferation and differentiation processes may overlap in time, as certain differentiation markers [6] are expressed during SC division. Nevertheless, SC numbers are essentially defined during the prepubertal period, so early perturbation of the proliferation and/or differentiation process can have long-term consequences on male reproduction.

It is well established that follicle-stimulating hormone (FSH) is mitogenic for immature SCs, both in vitro and in vivo [7–11]. A number of facts suggest that SC proliferation arrest and differentiation depend mainly on thyroid hormone T3 (T4 being the prohormone). In humans, neonatal hypothyroidism leads to testicular enlargement in prepubertal boys [12]. Neonatal hypothyroidism in mice and rats leads to an increase in the weight of adult testes and epididymides due to an increase in daily sperm production [13–18], while hyperthyroidism modulates the initiation of spermatogenesis [19]. In rodents, the postnatal period that marks the arrest of SC proliferation and entry into differentiation is marked by a peak in T3 blood levels [20], which also promotes brain maturation, bone growth, intestinal modeling, and several other developmental processes that are reminiscent of frog metamorphosis [21]. T3 acts, at least in large part, by binding to nuclear receptors TRα1, TRβ1, and TRβ2 (TRs), encoded by the two *THRA* and *THRB* genes that are found in all

tissues at various levels. TRs are transcription factors that bind to response elements, mainly as heterodimers with RXR nuclear receptors, in a ligand-independent manner. T3 binding induces conformational changes in the C-terminal domain of TRs, which entails the release of transcription corepressors and the recruitment of coactivators. Hypothyroidism mainly appears to be the result of transcriptional repression by unliganded TRs rather than the absence of transactivation.

Homozygous mice with null deletion of the *THRA* gene ($TR\alpha^{0/0}$) exhibit increased testicular weights in comparison with the wild-type (WT), and a tendency toward higher daily sperm production and SC numbers [22] in adults, which points to a predominant function of $TR\alpha 1$ in postnatal SC development. It is unclear whether T3 acts directly on developmental processes, or indirectly by modifying the serum content or cellular interactions within tissues. Indirect effects are certainly at work that might affect SC proliferation and differentiation. Neonatal hypothyroidism can significantly modify circulating luteinizing hormone and FSH levels [23]. It also decreases Leydig cell numbers and activity, as well as plasma testosterone levels [24]. Separating the direct and indirect consequences of T3 deficiency, or *THRA* knockout, on SC development implies generating somatic mutations restricted to selected cell types. To this end, we generated new mouse models where Cre/loxP recombination enabled a mutant $TR\alpha 1$, the $TR\alpha^{AM1}$ receptor [25], to be expressed in SCs only using the AMH-cre line [26]. The mutation prevents interaction with coactivators, while interaction with corepressors is maintained. Transcription of T3-regulated genes is thus constitutive, and the mutation is dominant negative. Phenotypic analysis demonstrated that T3 limits SC proliferation mainly by acting in a cell-autonomous manner.

MATERIALS AND METHODS

Animals

Mice were fed a standard laboratory diet and tap water ad libitum and maintained under 12L:12D photoperiods in a temperature-controlled room at 21–23°C. All animal studies were conducted in accordance with the guidelines for the care and use of laboratory animals issued by the French Ministry of Agriculture and with the approval of a local ethical review committee (number 2011-09-10 [Comité d'Éthique en Expérimentation Animale Val de Loire]).

Two lines were analyzed in the present study:

1) We generated and analyzed the $TR\alpha^{AM1}$ -SC line (for $TR\alpha^{AM1}$ allele targeted in SCs), which exhibits a targeted dominant-negative $TR\alpha 1$ isoform in SCs, using the CRE/Lox recombination system. The $TR\alpha^{AM1}$ allele for the *THRA* gene encodes a $TR\alpha 1$ receptor, $TR\alpha 1^{L400R}$, with an AF-2 mutation that corresponds to a point mutation changing a leucine to an arginine (L400R). This mutation prevents the recruitment of histone acetyltransferase coactivators, while preserving interaction with histone deacetylase corepressors, and results in dominant-negative activity (repression of transcription maintained), even in the presence of T3. Cre recombination is required to express the mutated allele, inactivating the $TR\alpha 1$ receptor (knockin). To obtain $TR\alpha^{AM1}$ -SC mice, we used $TR\alpha^{AM1/AM1}$ mice generated and characterized by Quignodon et al. [25] and previously used to express the dominant-negative mutation in cerebellum [27]. We crossed homozygous $TR\alpha^{AM1/AM1}$ mice with AMH-Cre mice [26] to obtain the animals of interest in the litter: $TR\alpha^{AM1/+}$ -Cre⁺, referred to as $TR\alpha^{AM1}$ -SC, and their adequate control mice (the same strategy as in the cerebellum study [27]), $TR\alpha^{AM1/+}$ -Cre⁻ (referred to as Cre⁻).

The AMH-Cre line induces Cre-mediated recombination in SCs, beginning at the age of 14.5 days postcoitum [26]. This line has been used for the functional study of various SC genes by targeted deletion [28–30].

2) We also analyzed the previously characterized $TR\alpha^{0/0}$ line with null deletion of the *THRA* gene. This line was generated by Gauthier et al. [31], and its male reproductive function was first described by Holsberger et al. [22]; the adequate control was the 129SV line.

PCR genotyping of $TR\alpha^{AM1}$, CRE, and $TR\alpha^{0/0}$ have been described previously [25, 26, 31].

Determination of Testis Weight and Sperm Reserve

$TR\alpha^{0/0}$ and $TR\alpha^{AM1}$ -SC and their respective control mice were sacrificed, and one testis was weighed and then frozen for sperm reserve determination, as described previously [32]. Briefly, testes were disrupted in 3 ml of L15 medium (Gibco-Invitrogen) in a glass potter before sonication for 30 sec. Remaining sperm nuclei were counted using hemocytometry. These nuclei contain spermatozoa and elongating spermatid nuclei (stages II–VII), and their number defines the testicular sperm reserve [18]. The testicular sperm reserve per milligram was obtained after dividing whole testicular sperm reserve by the weight of the testis.

Fertility Test

Each male (n = 10 per genotype) was mated with two primiparous Swiss females; birth date were noted to detect a putative delay in mating, and pups were counted at birth.

Plasma Testosterone

Mice were treated with an intraperitoneal injection of 15 IU/animal of human chorionic gonadotropin (hCG) (Chorulon). Blood was collected before (basal level) and 2 h after this injection (stimulated level). The plasma was stored at –20°C until tritium-based testosterone competitive radioimmunoassays were carried out as described previously [33]. The sensitivity of the assay was 0.125 ng/ml and the intra-assay coefficient of variation was 7.5%. Briefly, samples (two dilutions per sample) or testosterone dilutions (to determine the range) were incubated for 1 h at 40°C (buffer: 0.1 M phosphate buffer 0.1% gelatin) with tritiated testosterone plus the anti-testosterone antibody. A secondary antibody was then added, and the mixtures incubated for one night at 4°C. Immunoprecipitation was then performed with polyethyleneglycol 4000, and radioactivity was measured (Packard C2900 TriCarb).

Determination of SC Proliferation Index In Vivo

$TR\alpha^{0/0}$ mice at P3 and $TR\alpha^{AM1}$ -SC mice at Postnatal Day (P) 0, P3, and P10 were injected with 50 µg/g of body weight of 5-bromo-2-deoxyuridine (BrdU) (Sigma) that was incorporated in proliferating cells 3 h before sacrifice. Testes were fixed in Bouin fluid, embedded in paraffin, and sectioned (4 µm). After antigen retrieval in a boiling sodium citrate buffer (pH 6.0), endogenous peroxidase activity was quenched with H₂O₂. BrdU immunodetection was performed using a mouse immunoglobulin G monoclonal antibody (Roche; 1/200), and revealed using a peroxidase-labeled polymer conjugated to goat anti-mouse immunoglobulin (Dako) and 3-3'-diaminobenzidine chromogen (Dako). Negative controls were processed without the BrdU antibody. After counterstaining with hematoxylin, a total of 1000 proliferating (BrdU-stained) and quiescent SCs were counted (blindly) per animal using Histolab (GT Vision) analysis software.

Organotypic Cultures of $TR\alpha^{0/0}$ and $TR\alpha^{AM1}$ -SC Testicular Explants

Testes of $TR\alpha^{0/0}$ and $TR\alpha^{AM1}$ mice at P3 were cut into small pieces for organotypic cultures on floating membranes. Culture conditions were as described previously [34, 35], except that T3 (0.2 µM) or a vehicle (1× PBS, 0.025 N NaOH) was added to the medium. Explants were cultured for 72 h and BrdU was added (at a final concentration of 0.01 mg/ml) after 69 h. The explants were then fixed in Bouin fixative for BrdU immunostaining.

Histology, Diameter, Surface Measurement, and Cell Counting

Testis histology (seminiferous tubule organization) was analyzed after fixing in Bouin fluid and embedding in paraffin. Sections (4 µm) were stained with hematoxylin for microscopic observation.

In order to measure the seminiferous tubule diameters and surface areas (using Morphostrider software), and to determine SC and round spermatid (RS) densities, testes (n = 4 per genotype) were fixed in 4% 0.1 M glutaraldehyde/cacodylate buffer, and further fixed in 1% cacodylate-buffered osmium tetroxide prior to being embedded in epoxy resin. Tissue sections of 1 µm were prepared and stained with toluidine blue.

Two testicular pieces were prepared per animal, and three series of tissue sections per piece (25 µm apart so as to avoid analyzing the same area) were processed. A total of 200 round tubules per animal were selected for diameter measurements. In parallel, 200 randomly chosen tubules were visualized per

TABLE 1. Quantitative PCR primer sequences and hybridization temperature.

Genes	qPCR primers sequences	Hybridization temp.
β -Actin reverse	5'TGA CCC AGA TCA TGT TTG AGA 3'	55°C
β -Actin forward	5'TAC GAC CAG AGG CAT ACA GG 3'	
JunD reverse	5'TCT TTT TGT TTG GTT TTG TTT TGC 3'	60°C
JunD forward	5'GCG AAC CAA GGA TTA CGG AA 3'	
c-myc reverse	5'CAG AGG AGG AAC GAG CTG AAG CGC 3'	60°C
c-myc forward	5'TTA TGC ACC AGA GTT TCG AAG CTG TTC G 3'	

animal: 1) to count SCs and RSs (stages I–VI of the seminiferous epithelium cycle [36]) inside each tubule and 2) to measure the surface area (μm^2) of each tubule. The SC and RS densities per unit area of tubule (cell number/ μm^2) were calculated using these parameters.

RNA Extraction and Quantitative RT-PCR of Testes at P3

Total RNA was isolated from whole testes at P3 using Trizol reagent (Invitrogen) according to the manufacturer's instructions.

To quantify the expression of genes involved in the cell cycle, we used Mouse Cell cycle RT² Profiler PCR Array technology (Qiagen; <http://www.sabiosciences.com/PCRRArrayPlate.php>) as recommended by the manufacturer and as used by others [37]. Gene expression levels were normalized using five reference genes in the array: β -glucuronidase, hypoxanthine guanine, heat shock protein 90ab1, glyceraldehyde-3-phosphate dehydrogenase, and β -actin. The comparative cycle threshold method was used to calculate the relative quantification of gene expression. The fold change, which represents the variation in the level of expression between TR $\alpha^{\text{AMI-SC}}$ and the control, was calculated using the Web-based RT² profiler PCR Array Data Analysis program. A list of differentially expressed genes was identified using a two-tailed *t*-test, with a *P* value < 0.05 (*t*-test) and a mean difference equal to or greater than 2-fold (top right area; see Fig. 6).

To determine JunD, c-myc and β -actin mRNA levels by quantitative RT-PCR (qRT-PCR), RT was performed using M-MLV-RT (Invitrogen) according to the manufacturer's recommendations. Quantitative RT-PCR reactions were performed using SYBR Green SuperMix (Bio-Rad) according to the manufacturer's instructions and run in triplicate in an iCycler (Bio-Rad). Specific primers and hybridization temperatures are indicated in Table 1. In order to control the differences in RNA concentrations between each sample, the transcript level of each target gene was normalized on the basis of the transcript levels of the constitutive housekeeping gene β -actin (once we had verified that β -actin mRNA levels were not significantly different between samples).

Statistical Analysis

All data are presented as means \pm SEM. To compare means between two groups, the Student *t* test, or the Mann-Whitney *U*-test in case of differences in variance (Fisher test), were used. In addition, SAS Software (SAS Institute Inc.) was used for the stereology study (SC and RS densities, seminiferous tubule diameter). This software allows a combined statistical process to be used that is useful with low numbers of animals, as random effects are taken into account.

Other comparisons were performed using a two-way ANOVA followed by the Bonferroni post test. *P* < 0.05 was considered significant. Statistical analyses for the interpretation of the "Volcano Plot" in Figure 6 (see details in the legend) were proposed by the Web-based RT² profiler PCR Array Data Analysis program.

RESULTS

Generation and Characterization of TR $\alpha^{\text{AMI-SC}}$ Mice

In the TR α^{AMI} allele, transcription is interrupted by a cassette flanked by loxP sequences (see Supplemental Figure S1; all Supplemental Data are available online at www.biolreprod.org). TR α^{L400R} expression therefore requires Cre-mediated recombination to eliminate the cassette. We crossed TR $\alpha^{\text{AMI/AMI}}$ mice with AMH-CRE mice to produce TR $\alpha^{\text{AMI/+}}$ /AMH-CRE mice, hereafter called TR $\alpha^{\text{AMI-SC}}$. The littermates carrying only one transgene and not expressing TR α^{L400R} were retained as adequate controls (hereafter called Cre⁻).

TR $\alpha^{\text{AMI-SC}}$ Testicular Phenotype Is Similar to That of TR $\alpha^{\text{0/0}}$ in Adulthood

At 4 mo, both TR $\alpha^{\text{AMI-SC}}$ and TR $\alpha^{\text{0/0}}$ (controls of which are referred to as WT) exhibited a significant increase in testis weight in comparison with the respective controls (Fig. 1a); no change in body weight was observed (TR $\alpha^{\text{0/0}}$, 26.4 \pm 0.8 g vs. WT, 26.2 \pm 0.8 g; TR $\alpha^{\text{AMI-SC}}$, 29.3 \pm 0.5 g vs. Cre⁻, 28.6 \pm 0.8 g). This increase in testis weight was probably the consequence of a significant increase in whole testicular sperm reserve (spermatozoa and stage II–VII elongating spermatids) (Fig. 1b). Moreover, a significant increase in testicular sperm reserve, expressed per milligram of testis, was observed in both lines (Fig. 1c). TR $\alpha^{\text{AMI-SC}}$ males were as fertile as their controls, with no difference in pup numbers (TR $\alpha^{\text{AMI-SC}}$, 12.9 \pm 0.8 pups vs. Cre⁻, 12.3 \pm 1.8 pups; *n* = 10 males per genotype) after mating and pregnancy of females. No qualitative histological alterations in seminiferous tubule structures were observed in TR $\alpha^{\text{AMI-SC}}$ (Fig. 2), with all germ cell differentiation stages being present. In order to investigate a putative effect on Leydig cell steroidogenic activity, we measured testosterone levels in two conditions: before (basal level) and after (stimulated level) injection of hCG. As expected, injection of hCG induced a significant increase in blood testosterone levels in all lines (Fig. 3). No significant difference between the lines was observed for the basal or stimulated levels.

Altogether, the results based on the above physiological, histological, and endocrinological investigations indicate that a testicular phenotype similar to that observed in adult TR $\alpha^{\text{0/0}}$ could be reproduced by the presence of a dominant-negative TR α 1 isoform in SCs.

Testicular Phenotype of Adult TR $\alpha^{\text{AMI-SC}}$ Is the Result of an Increase in the SC Proliferation Index During Postnatal Development

To investigate further the testicular phenotype in adult TR $\alpha^{\text{AMI-SC}}$, we examined the SC density in seminiferous tubules (see details in *Materials and Methods*). At the age of 4 mo, TR $\alpha^{\text{AMI-SC}}$ exhibited an increase in SC density in comparison with the control mice (Table 2). This difference was significant according to the Mann-Whitney *U*-test, yet large but not significant (*P* = 0.06) when random effects were taken into account using the SAS combined process. In contrast, the RS density (stages I–VI of the seminiferous epithelium cycle) did not change between TR $\alpha^{\text{AMI-SC}}$ and Cre⁻ (Table 2). The RS/SC ratio was 6.34 and 7.97 for TR $\alpha^{\text{AMI-SC}}$ and controls, respectively. Moreover, according to both tests, a significant decrease in diameter of the seminiferous tubules was observed for TR $\alpha^{\text{AMI-SC}}$ in comparison with the controls (Table 2). To explain the increase in SC density in adults, we evaluated the proliferation rate of these cells in the young (Fig. 4). The percentage of SCs incorporating BrdU

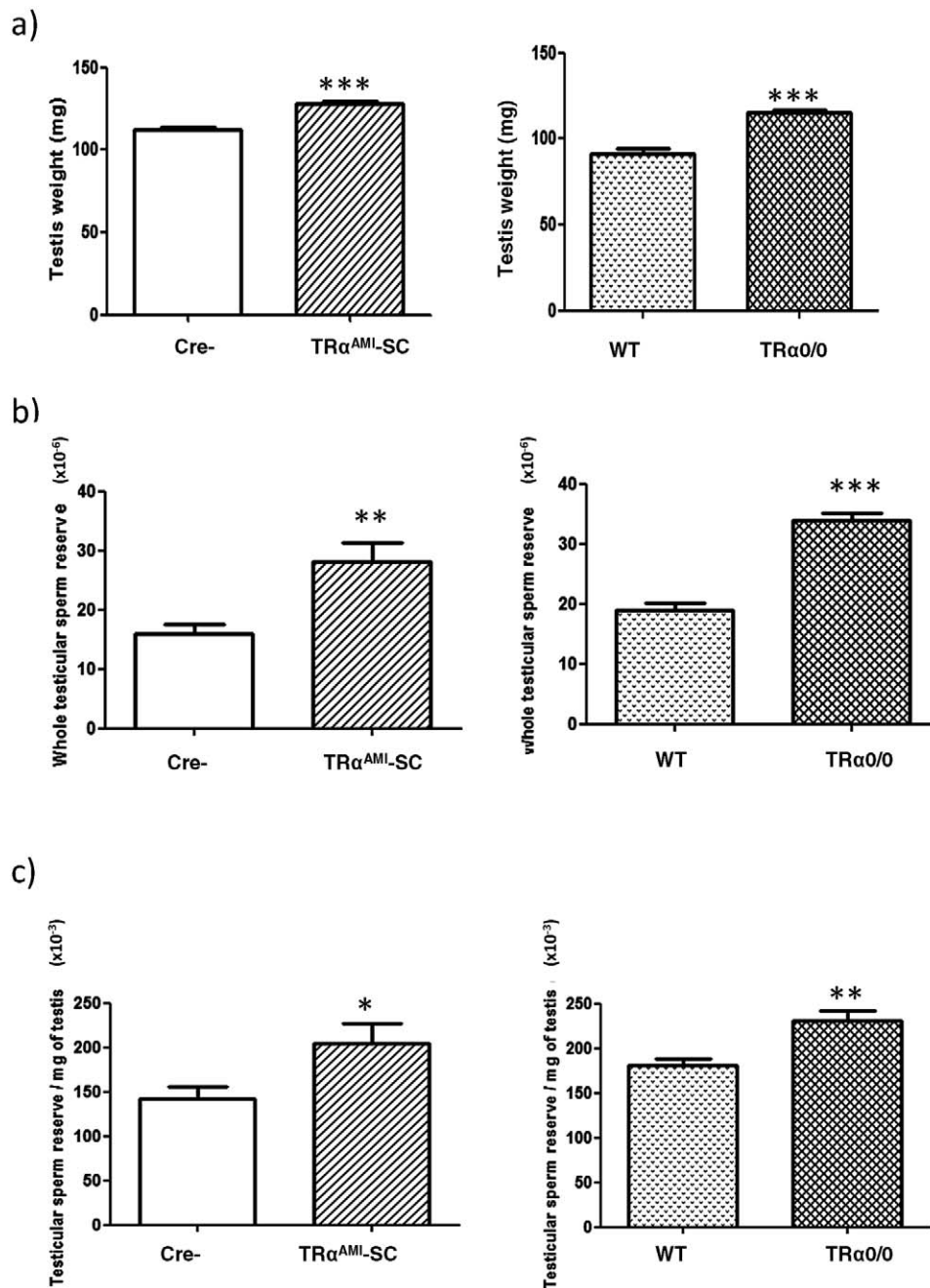


FIG. 1. Testis weight (a), whole testicular sperm reserve (b), and testicular sperm reserve per milligram of testis (c) in adult TR α^{AML-SC} and TR $\alpha^{0/0}$ mice (4 mo). A significant increase in testis weight (a) was observed in TR α^{AML-SC} and TR $\alpha^{0/0}$ mice in comparison with the controls (*** $P < 0.001$; $n = 20$ for Cre $^{-}$ and $n = 28$ for TR α^{AML-SC} ; $n = 12$ for WT and TR $\alpha^{0/0}$). Whole testicular sperm reserve (b) and sperm reserve expressed per milligram of testis (c) significantly increased in TR α^{AML-SC} (** $P < 0.01$ and * $P < 0.05$, respectively), and in TR $\alpha^{0/0}$ (*** $P < 0.001$ and ** $P < 0.01$, respectively). Data are shown as the mean \pm SEM and statistical analyses were performed using the *t*-test (independently for each graph). White bars, Cre $^{-}$; diagonal bars, TR α^{AML-SC} ; dotted bars, WT; hatched bars, TR $\alpha^{0/0}$.

within 3 h (Fig. 4a) was evaluated in TR α^{AML-SC} at P0, P3, and P10, and in TR $\alpha^{0/0}$ at P3. The SC proliferation index was significantly higher in TR α^{AML-SC} testes than in Cre $^{-}$ controls at P0 and P3, whereas, at P10, it was similar to the controls (Fig. 4b). A similar increase in the SC proliferation index was observed for TR $\alpha^{0/0}$ at P3 in comparison with WT. Both control lines (Cre $^{-}$ and WT 129SV) had similar proliferation indices. Altogether, from these results, it can be concluded that: 1) the phenotype in adult TR α^{AML-SC} originated from the increased SC proliferation index in the young before P10 and 2) as SCs had similar proliferative features in both lines, the

testicular phenotype in TR $\alpha^{0/0}$ can be explained by the SC deficit in this line.

The Increase in SC Proliferation in TR α^{AML-SC} and TR $\alpha^{0/0}$ Mediated by TR $\alpha 1$ During Postnatal Development Is T3 Dependent

We wished to investigate if the control exerted by TR $\alpha 1$ on the SC proliferation index described above was modulated in the presence of T3. To do so, we used organotypic cultures in which SC proliferation was measured in TR $\alpha^{0/0}$ and TR α^{AML-SC} .

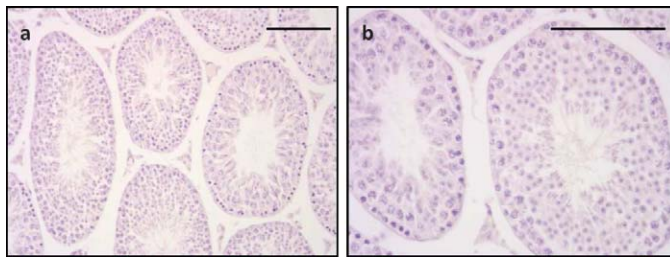


FIG. 2. Histological sections of adult TR α^{AM1} -SC testes at low (a) and high (b) magnifications (hematoxylin staining). Seminiferous tubules were fully and normally developed in TR α^{AM1} -SC, with their epithelium exhibiting normal structure and organization. Bar = 100 μ m.

SC testes and their respective controls explanted at P3, and in the presence or absence of exogenous T3 (Fig. 5). The advantage of this system was that local effects could be observed without interference from putative systemic effects (which would be observed in the case of *in vivo* treatment).

SC proliferation in cultures without T3 (vehicle) was similar between TR α^{AM1} -SC and Cre $^{-}$ testes (Fig. 5a) or between TR $\alpha^{0/0}$ and WT testes (Fig. 5b), indicating that the TR $\alpha 1$ dominant-negative isoform or null deletion did not alter SC proliferation in the absence of T3. We believe that this result may be due to the fact that the lack of T3 in the basal medium provides an environment similar to hypothyroidism *in vivo* in which no additional effect of the loss of the receptor would be expected. In both Cre $^{-}$ (Fig. 5a) and WT (Fig. 5b) testes, T3 significantly decreased SC proliferation as expected, whereas this response was abolished in both TR $\alpha^{0/0}$ and TR α^{AM1} -SC. Both mutants maintained a proliferation rate similar to that observed with the vehicle. This loss of response to T3 in both mutants indicates that T3 acts directly on the testes, independently of any other somatic effects, and that SC response is probably cell autonomous, which explains the phenotype observed *in vivo*.

Cell Cycle Gene Expression Is Altered in TR α^{AM1} -SC Testes at P3

In order to highlight actors in the cell cycle putatively involved in the increase in SC proliferation observed in

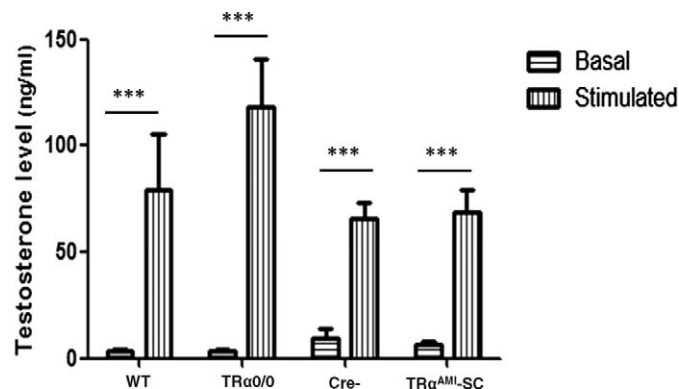


FIG. 3. Blood testosterone levels in TR α^{AM1} -SC and TR $\alpha^{0/0}$ mice, in basal conditions and after hCG stimulation, compared to the respective control mice. No significant difference between the lines was observed for the basal and stimulated levels; hCG induced a significant increase ($***P < 0.001$) in testosterone secretion, and this increase was similar in all lines. Data are shown as the mean \pm SEM; statistical analyses: two-way ANOVA followed by Bonferroni post test ($n = 26$ for Cre $^{-}$ and $n = 16$ for TR α^{AM1} -SC; $n = 9$ for WT and TR $\alpha^{0/0}$).

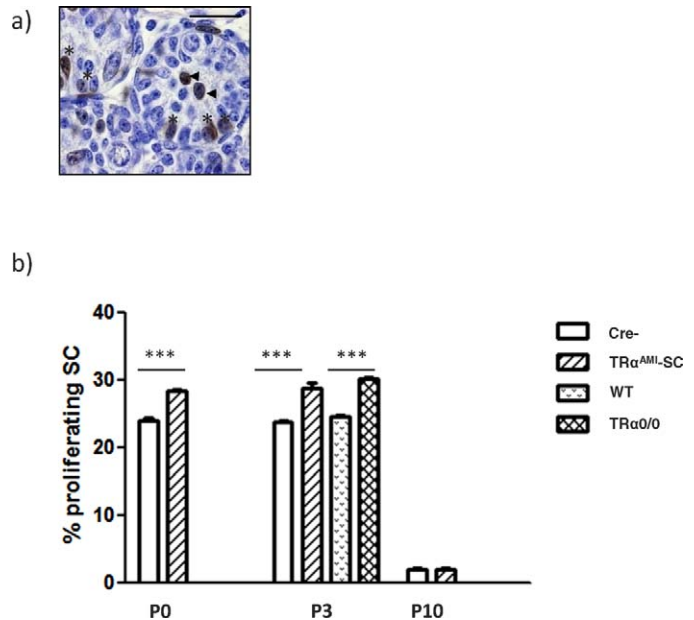


FIG. 4. Percentage of *in vivo* proliferating SCs in TR α^{AM1} -SC (at P0, P3, and P10) and in TR $\alpha^{0/0}$ (at P3). a) Immunohistochemical labeling of proliferating SC (stars) and gonocytes (arrowheads) revealed by BrdU incorporation injected 3 h before death in P3 testes of TR α^{AM1} -SC. Bar = 20 μ m. b) BrdU-negative and -positive SCs were counted and SC proliferation index was calculated. In TR α^{AM1} -SC testes, proliferation of SCs significantly increased in P0 and P3 testes in comparison with the control ($***P < 0.001$), whereas at P10 there was no longer any difference ($n = 4$ /genotype/age). In TR $\alpha^{0/0}$ testes at P3 ($n = 6$), proliferation of SCs was significantly higher than in the control ($n = 7$; $***P < 0.001$). Data are shown as the mean \pm SEM; statistical analyses was by two-way ANOVA followed by Bonferroni post test.

TR α^{AM1} -SC at P3, we analyzed 84 candidate genes (Supplemental Table S1), which are known to be involved in the cell cycle, using the RT 2 Profiler PCR Array dedicated to the mouse cell cycle. In TR α^{AM1} -SC testes at P3, we found four significantly upregulated genes (Fig. 6, triangles; two-tailed *t*-test; $P < 0.05$; main difference ≥ 2 -fold): Cdk4 (cyclin-dependent kinase 4; 27.99 fold), Sfn (stratifin; 4.36 fold), Apbb1 (amyloid beta A4 precursor protein-binding, family B, member 1; 3.42 fold) and Cdkn2a (cyclin-dependent kinase inhibitor 2A; 2.84 fold). No downregulated genes were found (Supplemental Table S1, first and second columns). Messenger RNA levels of cyclins, Cdk, or Cdkn, including Cdkn1b (p27 kip1) and Cdkn1a (p21), were unchanged. These results were observed using “classical” qRT-PCR with other validated primers for Cdkn1b and Cdkn1a (data not shown).

It is known that Cdk4 transcription is controlled by two main regulators: JunD (repressor [38, 39]) and c-myc (activator [40, 41]), of which the mRNA was not present in the array. So, to consolidate our finding regarding the involvement of Cdk4, we measured the mRNA levels of JunD and c-myc in P3 TR α^{AM1} -SC testes using qRT-PCR with specific primers (Table 1). A 3-fold decrease in JunD mRNA (Fig. 7a) and a 2-fold increase in c-myc mRNA (Fig. 7b) were observed.

DISCUSSION

TR α^{AM1} -SC and TR $\alpha^{0/0}$ Exhibit the Same Testicular Phenotype

TR α^{AM1} -SC mice, where the dominant-negative TR $\alpha 1^{L400R}$ mutation is expressed in SCs, display a testis phenotype that is

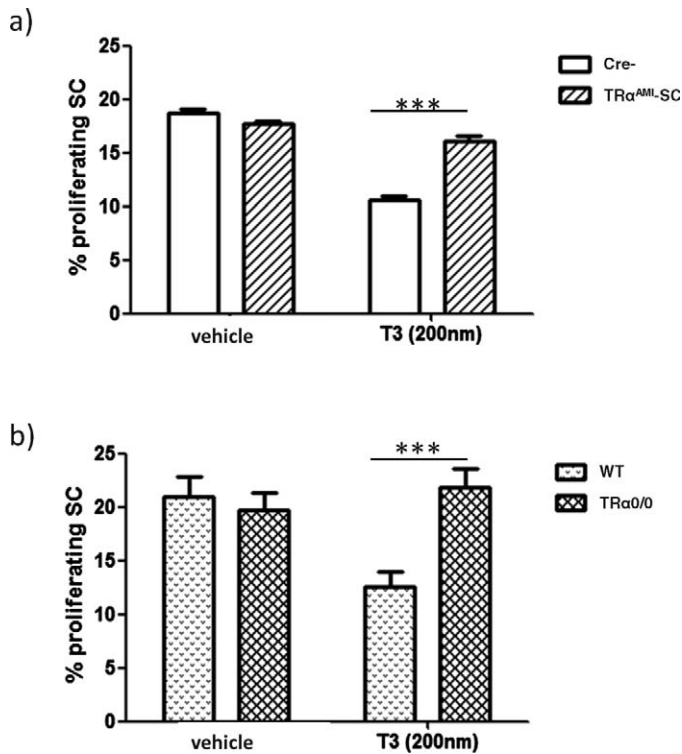


FIG. 5. SC proliferation index in testicular explants from TR α^{AML-SC} and TR $\alpha^{0/0}$ mice at P3, using organotypic cultures with or without exogenous T3. Organotypic cultures of P3 testes from TR α^{AML-SC} ($n = 6$ for controls and $n = 5$ for TR α^{AML-SC} [a]) and TR $\alpha^{0/0}$ ($n = 4$ for WT and TR $\alpha^{0/0}$ [b]) mice were performed with T3 (200 nM) or vehicle (PBS 1 \times , 0.025 N NaOH). After 3 days of culture, BrdU was added 3 h before explant fixation. SC proliferation was evaluated as with in vivo proliferation. The SC proliferation index significantly decreased in both controls in the presence of T3 compared with vehicle (** $P < 0.001$). In contrast, T3 had no effect on the SC proliferative index in TR α^{AML-SC} and TR $\alpha^{0/0}$ mice. Data are shown as the mean \pm SEM; statistical analyses was by two-way ANOVA followed by Bonferroni post test.

very similar to the phenotype of TR $\alpha^{0/0}$ knockout mice, with increased testicular sperm reserve and testis weight.

To investigate further the TR α^{AML-SC} testicular phenotype in adulthood, we performed SC and RS counts to determine the densities (cell number/ μm^2). We observed that the SC density increased in TR α^{AML-SC} testes in comparison with the controls, while the RS density remained unchanged. The increase in the SC density is in accordance with the tendency toward an increase in SC numbers described in TR $\alpha^{0/0}$ testes [22]. As a consequence of the increase in SC density and the unchanged RS density, the number of RSs per SC decreased from 7.97 in Cre $^{-}$ to 6.34 in TR α^{AML-SC} , which corresponds to a decrease of 20.5% ($100 \times [7.97 - 6.34]/7.97$). The decrease in the RS/SC ratio reflects the attenuated ability of SCs to support a normal number of germ cells during differentiation. This is in complete agreement with what has been described in adult rats after neonatal hypothyroidism by Hess et al. [42], where the germ cell differentiation yield decreased by 28%, but was compensated by an increase in sperm production and the total number of SCs. We also observed a decrease in the diameter of the seminiferous tubules in adult TR α^{AML-SC} in comparison with the controls, as observed in TR $\alpha^{0/0}$ at P10 [22]. This could be the consequence of a shrinking process due to spatial competition between SC.

Another interesting result from our study was obtained using the organotypic culture system that enabled us to observe the effect of molecules directly on testes cells, with no interference from central or endocrine regulation. When Holsberger et al. [22] injected T3 in vivo at P10, they observed the result of local testicular and central/endocrine T3 perturbations, and this probably explains why the response to T3 that they observed was only partially abolished. In contrast, at P3, when we added T3 to testis explants, the decrease in the SC proliferation index observed in control mice (similar to that observed above in vivo) was totally abolished in both TR α^{AML-SC} and TR $\alpha^{0/0}$ mice.

In vivo, at P3, the SC proliferation index was significantly higher in both TR α^{AML-SC} and TR $\alpha^{0/0}$ mice than in their respective controls. SC proliferation is characterized by two parameters: the rate of proliferation and the length of the postnatal period during which SC actively proliferate. Our results suggest that SC TR $\alpha 1$ impacts the proliferation rate rather than the length of the proliferative phase. This is corroborated by the fact that testicular TR $\alpha 1$ is expressed from birth in rodents [43, 44]. In neonate propylthiouracil-treated mice, an extension of the proliferative period (to P20) was observed [18]. In the latter model, all the TRs in the various organs of the hypothalamo-pituitary-testis axis were “affected” by hypothyroidism; thus, the extension of the proliferative phase described may involve either other TRs or systemic feedback regulation.

Altogether, the phenotypes described here are a strong indication that, in a physiological situation, T3 controls mainly postnatal SC development by binding to TR $\alpha 1$, acting in a cell-autonomous manner during the early postnatal period, as after postnatal hypothyroidism, the SC proliferation defect has long-term consequences. Although our data do not rule out other modes of action, such as so-called nongenomic signaling [45] and the intervention of the mitochondrial targeted isoform [46], they establish the essential role of the *THRA* gene in this process.

A Predominant Function of THRA over THRB for SC Development

Although the convergence of the SC phenotype in TR α^{AML-SC} and TR $\alpha^{0/0}$ is a strong indication that TR $\beta 1$ has no function in SC development, this possibility cannot be ruled out completely. First, TR $\beta 1$, of which the expression in rat testes is controversial [44, 47], might act indirectly on the SC environment. In fact, in our models, we did not observe a decrease in testosterone levels [24] or an extension of the SC proliferative period that has been reported after postnatal hypothyroidism in rats [18]. The fact that the TR $\alpha^{0/0}$ testis phenotype does not fully recapitulate postnatal hypothyroidism might be an indication that TR β fulfills certain functions of some indirect T3 functions. In particular, TR β could be acting in Leydig cells, maintaining testosterone levels. Second, if TR β has a cell-autonomous function in SC during postnatal development, this might be inhibited by TR $\alpha 1^{L400R}$, as the two isoforms can cross-talk, at least in vitro. However, the systematic analysis of mice expressing TR $\alpha 1^{L400R}$ in all tissues suggests that most TR β functions are preserved in this model [25, 27].

Cell Cycle Actors Involved in SC Proliferation in TR α^{AML-SC} and TR $\alpha^{0/0}$

Gene expression profiling in testes at P3 highlighted changes in the expression of several genes in TR α^{AML-SC}

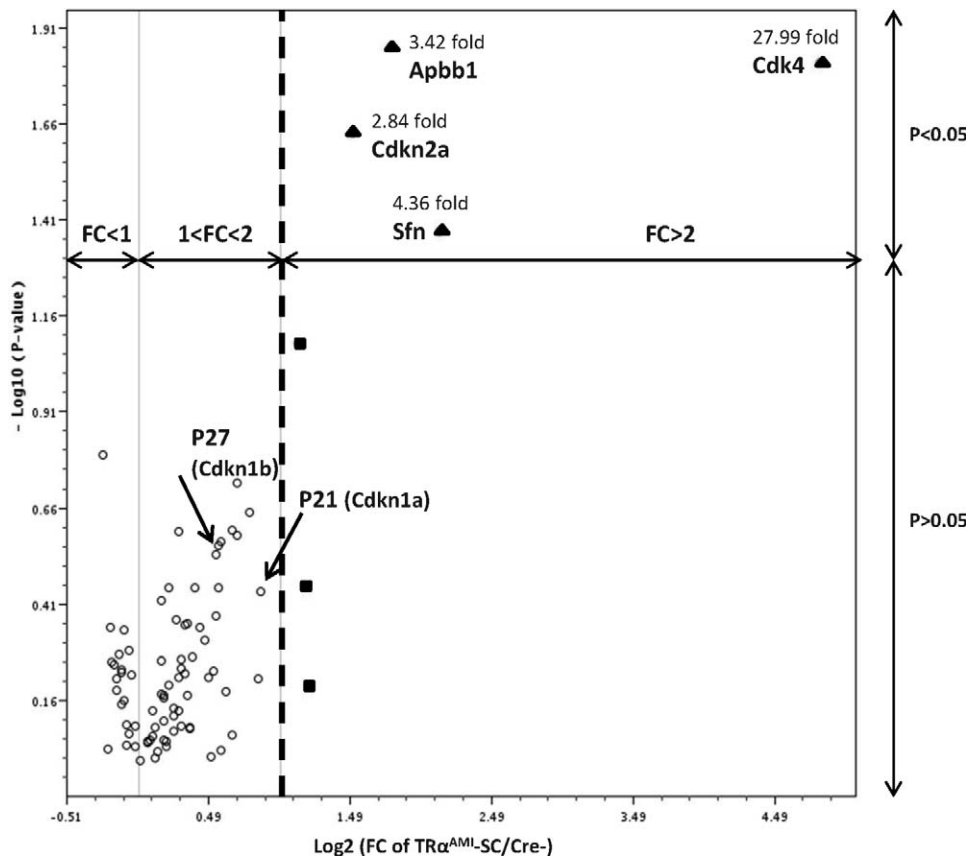


FIG. 6. Cell cycle gene expression is impacted in TR α^{AML} -SC testes at P3. The “Volcano Plot” is an arbitrary representation (proposed by the Web-based RT² profiler PCR Array Data Analysis program) of the fold change (FC) for each of the 84 genes in the array. It represents the Log₂ FC of each gene expression between the TR α^{AML} -SC group (n = 6) and the control group (n = 6) versus the negative Log₁₀ P values from the t-test. The first vertical line indicates fold changes of 1 (no variation between groups). The second vertical line (dotted line) indicates that the gene expression fold change threshold is 2. The horizontal line indicates that the P value of the t-test threshold is 0.05. There were four genes (triangles) that were significantly upregulated in TR α^{AML} -SC in comparison with the controls.

mice that might provide some clues as to the molecular mechanisms underlying the ability of T3 to arrest SC proliferation. In particular, the Cdk4/JunD/c-myc pathway was clearly altered. As far as we know, this is the first time that CDK4 upregulation has been evoked in proliferating SC. Interestingly, studies of Cdk4^{-/-} mice [48] have revealed growth retardation and reproductive dysfunction associated with testicular atrophy and hypoplastic seminiferous tubules in the testes. The same pathway was also affected in TR $\alpha^{0/0}$ mice. However, in this model, there were numerous changes in genomic expression (see Supplemental Table S1, third and fourth columns: 14 genes significantly changed including Cdk4, Sfn, Apbb1, and Cdkn2a) that may be the indirect consequence of other levels of perturbations (germline mutations) and/or feedback responses. Increased Cdkn1b (p27^{kip1}) protein levels were reported in hyperthyroid SCs at P10 [49], and this response to excess T3 was lost in TR $\alpha^{0/0}$

[22]. However, in agreement with our current observation, the basal level was the same as in the controls [22]. Whether or not the changes in gene expression described here reflect direct transcriptional regulation of some of these genes by liganded TR α 1 remains to be determined.

Putative Consequences of THRA Function in SC Proliferation on Human and Animal Health

Thyroid hormone physiology in humans exhibits different features in comparison with rodents. For example, the T3/T4 half-life is considerably higher in humans than in rodents and developmental events occur in utero in humans and postnatally in rodents. However, the role of T3 in male reproduction and reproductive tract development is similar to what has been described in animals (for review: [12, 50]).

TABLE 2. SC density (SCs/ μ m²), RS density (RSs/ μ m²), and seminiferous tubule diameter in adult TR α^{AML} -SC and Cre⁻ (4 animals per genotype and 200 seminiferous tubules/animal).*

Parameter	Cre ⁻	TR α^{AML} -SC	Mann-Whitney U-test	SAS combined process
SC/ μ m ²	0.78 10 ⁻³ ± 0.01 10 ⁻³	0.96 10 ⁻³ ± 0.02 10 ⁻³	P < 0.001	P = 0.06
RS/ μ m ²	6.21 10 ⁻³ ± 0.05 10 ⁻³	5.90 10 ⁻³ ± 0.08 10 ⁻³	P = 0.11	P = 0.75
Diameter (μ m ²)	148.3 ± 0.9	135.5 ± 1.1	P < 0.001	P < 0.001

* Data are shown as the mean ± SEM, and statistical analyses were performed using the Mann-Whitney U-test or SAS combined process (see *Materials and Methods*).

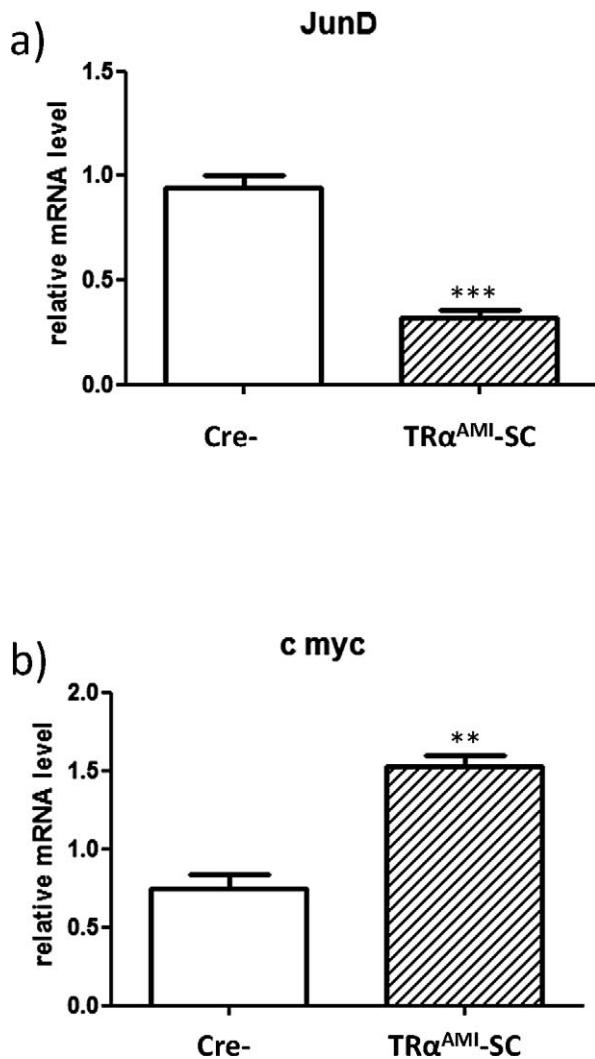


FIG. 7. JunD and c-myc gene expression is impacted in TR α^{AMI-SC} testes at P3. As JunD (a) and c-myc (b) were not present in the array, qRT-PCR analyses for these genes were performed with TR α^{AMI-SC} and control testes at P3 ($n = 6$ for each group) using specific primers (see Table 1). Normalization was achieved using β -actin levels. JunD mRNA levels significantly decreased ($***P < 0.001$) and c-myc levels increased ($**P < 0.01$). Data are shown as the mean \pm SEM, and statistical analyses were performed using the Student t test. White bars, Cre $^{-}$; diagonal bars, TR α^{AMI-SC} .

The fact that TR $\alpha 1$ is the predominant isoform involved in the arrest of SC proliferation has numerous consequences on human health. In particular, a number of chemical compounds present in our environment are suspected of altering T3 signaling, and it has been proposed that some of them, such as poly-chloro-biphenyls and poly-bromo-diphenyl-esters, act as TR antagonists [51–53]. Interestingly, a key element in defining these so-called thyroid hormone disruptors is their ability to alter the circulating levels of T4 and T3. As the preservation of T3 levels is mainly a TR β function, any antagonist compound displaying a marked preference for TR $\alpha 1$ binding would probably be overlooked in most assays. Our results, however, suggest that early exposure to a hypothetical compound such as this would alter SC differentiation and contribute to compromising male fertility.

ACKNOWLEDGMENT

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work. The English was proofread and corrected by Helen Lamprell from HSB Traductions. The authors would like to thank I. Fontaine, B. Delaleu, and T. Delpuech for their routine technical help; C. Duquenne, S. Moreno, and S. Messiaen for their advice in immunohistochemistry; and C. Cahier for animal care; K. Gauthier, M. Govoroun, and P. Monget for their helpful discussions.

REFERENCES

- Orth JM, Gonsalus GL, Lamperti AA. Evidence from Sertoli cell-depleted rats indicates that spermatid number in adults depends on numbers of Sertoli cells produced during perinatal development. *Endocrinology* 1988; 122:787–794.
- Steinberger A, Steinberger E. Replication pattern of Sertoli cells in maturing rat testis in vivo and in organ culture. *Biol Reprod* 1971; 4: 84–87.
- Nagy F. An autoradiographic study of the post-natal changes in (3H)leucine incorporation by Sertoli cell nuclei in rat testes. *J Reprod Fertil* 1974; 37:353–359.
- Vitale R, Fawcett DW, Dym M. The normal development of the blood-testis barrier and the effects of clomiphene and estrogen treatment. *Anat Rec* 1973; 176:331–344.
- Ahmed EA, Barten-van Rijbroek AD, Kal HB, Sadri-Ardekani H, Mizrak SC, van Pelt AM, de Rooij DG. Proliferative activity in vitro and DNA repair indicate that adult mouse and human Sertoli cells are not terminally differentiated, quiescent cells. *Biol Reprod* 2009; 80:1084–1091.
- Mazaud-Guittot S, Meugnier E, Pesenti S, Wu X, Vidal H, Gow A, Le Magueresse-Battistoni B. Claudin 11 deficiency in mice results in loss of the Sertoli cell epithelial phenotype in the testis. *Biol Reprod* 2010; 82: 202–213.
- Griswold M, Mably E, Fritz IB. Stimulation by follicle stimulating hormone and dibutyryl cyclic AMP of incorporation of 3H-thymidine into nuclear DNA of cultured Sertoli cell-enriched preparations from immature rats. *Curr Top Mol Endocrinol* 1975; 2:413–420.
- Griswold MD, Solari A, Tung PS, Fritz IB. Stimulation by follicle-stimulating hormone of DNA synthesis and of mitosis in cultured Sertoli cells prepared from testes of immature rats. *Mol Cell Endocrinol* 1977; 7: 151–165.
- Orth JM. The role of follicle-stimulating hormone in controlling Sertoli cell proliferation in testes of fetal rats. *Endocrinology* 1984; 115: 1248–1255.
- Meachem SJ, McLachlan RI, de Kretser DM, Robertson DM, Wreford NG. Neonatal exposure of rats to recombinant follicle stimulating hormone increases adult Sertoli and spermatogenic cell numbers. *Biol Reprod* 1996; 54:36–44.
- Singh J, Handelsman DJ. Neonatal administration of FSH increases Sertoli cell numbers and spermatogenesis in gonadotropin-deficient (hpg) mice. *J Endocrinol* 1996; 151:37–48.
- Jannini EA, Ulisse S, D'Armiento M. Thyroid hormone and male gonadal function. *Endocr Rev* 1995; 16:443–459.
- Cooke PS, Hess RA, Porcelli J, Meisami E. Increased sperm production in adult rats after transient neonatal hypothyroidism. *Endocrinology* 1991; 129:244–248.
- Cooke PS, Zhao YD, Bunick D. Triiodothyronine inhibits proliferation and stimulates differentiation of cultured neonatal Sertoli cells: possible mechanism for increased adult testis weight and sperm production induced by neonatal goitrogen treatment. *Biol Reprod* 1994; 51:1000–1005.
- van Haaster LH, de Jong FH, Docter R, de Rooij DG. High neonatal triiodothyronine levels reduce the period of Sertoli cell proliferation and accelerate tubular lumen formation in the rat testis, and increase serum inhibin levels. *Endocrinology* 1993; 133:755–760.
- Cooke PS, Meisami E. Early hypothyroidism in rats causes increased adult testis and reproductive organ size but does not change testosterone levels. *Endocrinology* 1991; 129:237–243.
- Palmero S, Prati M, Bolla F, Fugassa E. Tri-iodothyronine directly affects rat Sertoli cell proliferation and differentiation. *J Endocrinol* 1995; 145: 355–362.
- Joyce KL, Porcelli J, Cooke PS. Neonatal goitrogen treatment increases adult testis size and sperm production in the mouse. *J Androl* 1993; 14: 448–455.
- Marchlewska K, Kula K, Walczak-Jedrzejowska R, Oszukowska E, Filipiak E, Slowikowska-Hilczner J. Role of FSH and triiodothyronine in Sertoli cell development expressed by formation of connexin 43-based gap junctions. *J Exp Zool A Ecol Genet Physiol* 2011; 315:329–336.

20. Hadj-Sahraoui N, Seugnet I, Ghorbel MT, Demeneix B. Hypothyroidism prolongs mitotic activity in the post-natal mouse brain. *Neurosci Lett* 2000; 280:79–82.
21. Kress E, Samarut J, Plateroti M. Thyroid hormones and the control of cell proliferation or cell differentiation: paradox or duality? *Mol Cell Endocrinol* 2009; 313:36–49.
22. Holsberger DR, Kiesewetter SE, Cooke PS. Regulation of neonatal Sertoli cell development by thyroid hormone receptor alpha1. *Biol Reprod* 2005; 73:396–403.
23. Kirby JD, Arambepola N, Porkka-Heiskanen T, Kirby YK, Rhoads ML, Nitta H, Jetton AE, Iwamoto G, Jackson GL, Turek FW, Cooke PS. Neonatal hypothyroidism permanently alters follicle-stimulating hormone and luteinizing hormone production in the male rat. *Endocrinology* 1997; 138:2713–2721.
24. Antony FF, Aruldas MM, Udhayakumar RC, Maran RR, Govindarajulu P. Inhibition of Leydig cell activity in vivo and in vitro in hypothyroid rats. *J Endocrinol* 1995; 144:293–300.
25. Quignodon L, Vincent S, Winter H, Samarut J, Flamant F. A point mutation in the activation function 2 domain of thyroid hormone receptor alpha1 expressed after CRE-mediated recombination partially recapitulates hypothyroidism. *Mol Endocrinol* 2007; 21:2350–2360.
26. Lecureuil C, Fontaine I, Crepieux P, Guillou F. Sertoli and granulosa cell-specific Cre recombinase activity in transgenic mice. *Genesis* 2002; 33:114–118.
27. Fauquier T, Romero E, Picou F, Chatonnet F, Nguyen XN, Quignodon L, Flamant F. Severe impairment of cerebellum development in mice expressing a dominant-negative mutation inactivating thyroid hormone receptor alpha1 isoform. *Dev Biol* 2011; 356:350–358.
28. De Gendt K, Swinnen JV, Saunders PT, Schoonjans L, Dewerchin M, Devos A, Tan K, Atanassova N, Claessens F, Lecureuil C, Heyns W, Carmeliet P, et al. A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. *Proc Natl Acad Sci U S A* 2004; 101:1327–1332.
29. Barrionuevo F, Georg I, Scherthan H, Lecureuil C, Guillou F, Wegner M, Scherer G. Testis cord differentiation after the sex determination stage is independent of Sox9 but fails in the combined absence of Sox9 and Sox8. *Dev Biol* 2009; 327:301–312.
30. Weider K, Bergmann M, Giese S, Guillou F, Failing K, Brehm R. Altered differentiation and clustering of Sertoli cells in transgenic mice showing a Sertoli cell specific knockout of the connexin 43 gene. *Differentiation* 2011; 82:38–49.
31. Gauthier K, Plateroti M, Harvey CB, Williams GR, Weiss RE, Refetoff S, Willott JF, Sundin V, Roux JP, Malaval L, Hara M, Samarut J, et al. Genetic analysis reveals different functions for the products of the thyroid hormone receptor alpha locus. *Mol Cell Biol* 2001; 21:4748–4760.
32. Lecureuil C, Staub C, Fouchecourt S, Maurel MC, Fontaine I, Martinat N, Gauthier C, Daudignon A, Delaleu B, Sow A, Jegou B, Guillou F. Transferrin overexpression alters testicular function in aged mice. *Mol Reprod Dev* 2007; 74:197–206.
33. Hochereau-de Reviere MT, Perreau C, Pisselet C, Fontaine I, Monet-Kuntz C. Comparisons of endocrinological and testis parameters in 18-month-old Ile de France and Romanov rams. *Domest Anim Endocrinol* 1990; 7:63–73.
34. Livera G, Delbes G, Pairault C, Rouiller-Fabre V, Habert R. Organotypic culture, a powerful model for studying rat and mouse fetal testis development. *Cell Tissue Res* 2006; 324:507–521.
35. Merlet J, Moreau E, Habert R, Racine C. Development of fetal testicular cells in androgen receptor deficient mice. *Cell Cycle* 2007; 6:2258–2262.
36. Oakberg EF. Duration of spermatogenesis in the mouse and timing of stages of the cycle of the seminiferous epithelium. *Am J Anat* 1956; 99:507–516.
37. Zhang Q, Xiao X, Feng K, Wang T, Li W, Yuan T, Sun X, Sun Q, Xiang H, Wang H. Berberine moderates glucose and lipid metabolism through multipathway mechanism. *Evid Based Complement Alternat Med* 2011; 2011.
38. Xiao L, Rao JN, Zou T, Liu L, Marasa BS, Chen J, Turner DJ, Passaniti A, Wang JY. Induced JunD in intestinal epithelial cells represses CDK4 transcription through its proximal promoter region following polyamine depletion. *Biochem J* 2007; 403:573–581.
39. Hernandez JM, Floyd DH, Weillbaeher KN, Green PL, Boris-Lawrie K. Multiple facets of junD gene expression are atypical among AP-1 family members. *Oncogene* 2008; 27:4757–4767.
40. Haas K, Staller P, Geisen C, Bartek J, Eilers M, Moroy T. Mutual requirement of CDK4 and Myc in malignant transformation: evidence for cyclin D1/CDK4 and p16INK4A as upstream regulators of Myc. *Oncogene* 1997; 15:179–192.
41. Hermeking H, Rago C, Schuhmacher M, Li Q, Barrett JF, Obaya AJ, O'Connell BC, Mateyak MK, Tam W, Kohlhuber F, Dang CV, Sedivy JM, et al. Identification of CDK4 as a target of c-MYC. *Proc Natl Acad Sci U S A* 2000; 97:2229–2234.
42. Hess RA, Cooke PS, Bunick D, Kirby JD. Adult testicular enlargement induced by neonatal hypothyroidism is accompanied by increased Sertoli and germ cell numbers. *Endocrinology* 1993; 132:2607–2613.
43. Jannini EA, Dolci S, Ulisse S, Nikodem VM. Developmental regulation of the thyroid hormone receptor alpha 1 mRNA expression in the rat testis. *Mol Endocrinol* 1994; 8:89–96.
44. Canale D, Agostini M, Giorgilli G, Caglieresi C, Scartabelli G, Nardini V, Jannini EA, Martino E, Pinchera A, Macchia E. Thyroid hormone receptors in neonatal, prepubertal, and adult rat testis. *J Androl* 2001; 22:284–288.
45. Zamoner A, Pessoa-Pureur R, Silva FR. Membrane-initiated actions of thyroid hormones on the male reproductive system. *Life Sci* 2011; 89:507–514.
46. Wrutniak-Cabello C, Casas F, Cabello G. Thyroid hormone action in mitochondria. *J Mol Endocrinol* 2001; 26:67–77.
47. Rao JN, Liang JY, Chakraborti P, Feng P. Effect of thyroid hormone on the development and gene expression of hormone receptors in rat testes in vivo. *J Endocrinol Invest* 2003; 26:435–443.
48. Tsutsui T, Hesabi B, Moons DS, Pandolfi PP, Hansel KS, Koff A, Kiyokawa H. Targeted disruption of CDK4 delays cell cycle entry with enhanced p27(Kip1) activity. *Mol Cell Biol* 1999; 19:7011–7019.
49. Holsberger DR, Jirawatnotai S, Kiyokawa H, Cooke PS. Thyroid hormone regulates the cell cycle inhibitor p27Kip1 in postnatal murine Sertoli cells. *Endocrinology* 2003; 144:3732–3738.
50. Choksi NY, Jahnke GD, St Hilaire C, Shelby M. Role of thyroid hormones in human and laboratory animal reproductive health. *Birth Defects Res B Dev Reprod Toxicol* 2003; 68:479–491.
51. Koibuchi N, Iwasaki T. Regulation of brain development by thyroid hormone and its modulation by environmental chemicals. *Endocr J* 2006; 53:295–303.
52. Zoeller RT, Bansal R, Parris C. Bisphenol-A, an environmental contaminant that acts as a thyroid hormone receptor antagonist in vitro, increases serum thyroxine, and alters RC3/neurogranin expression in the developing rat brain. *Endocrinology* 2005; 146:607–612.
53. Zoeller RT. Environmental chemicals as thyroid hormone analogues: new studies indicate that thyroid hormone receptors are targets of industrial chemicals? *Mol Cell Endocrinol* 2005; 242:10–15.