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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]

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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^{AMI}) in SCs only. So-called TRalpha^{AMI}-SC (TRalpha$^{AMI/+}$ Cre^{+}) mice exhibited similar phenotypic features to the knockout line: heavier testicular weight and higher sperm reserve, in comparison with their adequate controls (TRalpha$^{AMI/+}$ 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^{AMI}-SC and knockout lines. Finally, when TRalpha^{AMI} 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

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

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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 TRα1 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 TRα1, the $TR\alpha^{AMI}$ 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'Ethique en Expérimentation Animale Val de Loire]).

Two lines were analyzed in the present study:

1) We generated and analyzed the $TR\alpha^{AMI}$-SC line (for $TR\alpha^{AMI}$ allele targeted in SCs), which exhibits a targeted dominant-negative TRα1 isoform in SCs, using the CRE/Lox recombination system. The $TR\alpha^{AMI}$ allele for the *THRA* gene encodes a TRα1 receptor, $TR\alpha1^{L400R}$, 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 TRα1 receptor (knockin). To obtain $TR\alpha^{AMI}$-SC mice, we used $TR\alpha^{AMI/AMI}$ mice generated and characterized by Quignodon et al. [25] and previously used to express the dominant-negative mutation in cerebellum [27]. We crossed homozygous $TR\alpha^{AMI/AMI}$ mice with AMH-Cre mice [26] to obtain the animals of interest in the litter: $TR\alpha^{AMI/+}$-Cre^{+}, referred to as $TR\alpha^{AMI}$-SC, and their adequate control mice (the same strategy as in the cerebellum study [27]), $TR\alpha^{AMI/+}$-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 $TR\alpha^{AMI}$, 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 $TR\alpha^{AMI}$-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 $TR\alpha^{AMI}$-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 H_2O_2. 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 $TR\alpha^{AMI}$-SC Testicular Explants

Testes of $TR\alpha^{0/0}$ and $TR\alpha^{AMI}$ 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 RT^2 Profiler PCR Array technology (Qiagen; http://www.sabiosciences.com/PCRArrayPlate.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 TRα^{AMI}-SC and the control, was calculated using the Web-based RT^2 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 ± 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 RT^2 profiler PCR Array Data Analysis program.

RESULTS

Generation and Characterization of TRα^{AMI}-SC Mice

In the TRα^{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). TRα1^{L400R} expression therefore requires Cre-mediated recombination to eliminate the cassette. We crossed TRα$^{AMI/AMI}$ mice with AMH-CRE mice to produce TRα$^{AMI/+}$/AMH-CRE mice, hereafter called TRα^{AMI}-SC. The littermates carrying only one transgene and not expressing TRα1^{L400R} were retained as adequate controls (hereafter called Cre$^-$).

TRα^{AMI}-SC Testicular Phenotype Is Similar to That of TRα$^{0/0}$ in Adulthood

At 4 mo, both TRα^{AMI}-SC and TRα$^{0/0}$ (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 (TRα$^{0/0}$, 26.4 ± 0.8 g vs. WT, 26.2 ± 0.8 g; TRα^{AMI}-SC, 29.3 ± 0.5 g vs. Cre$^-$, 28.6 ± 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). TRα^{AMI}-SC males were as fertile as their controls, with no difference in pup numbers (TRα^{AMI}-SC, 12.9 ± 0.8 pups vs. Cre$^-$, 12.3 ± 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 TRα^{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 TRα$^{0/0}$ could be reproduced by the presence of a dominant-negative TRα1 isoform in SCs.

Testicular Phenotype of Adult TRα^{AMI}-SC Is the Result of an Increase in the SC Proliferation Index During Postnatal Development

To investigate further the testicular phenotype in adult TRα^{AMI}-SC, we examined the SC density in seminiferous tubules (see details in *Materials and Methods*). At the age of 4 mo, TRα^{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 TRα^{AMI}-SC and Cre– (Table 2). The RS/SC ratio was 6.34 and 7.97 for TRα^{AMI}-SC and controls, respectively. Moreover, according to both tests, a significant decrease in diameter of the seminiferous tubules was observed for TRα^{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

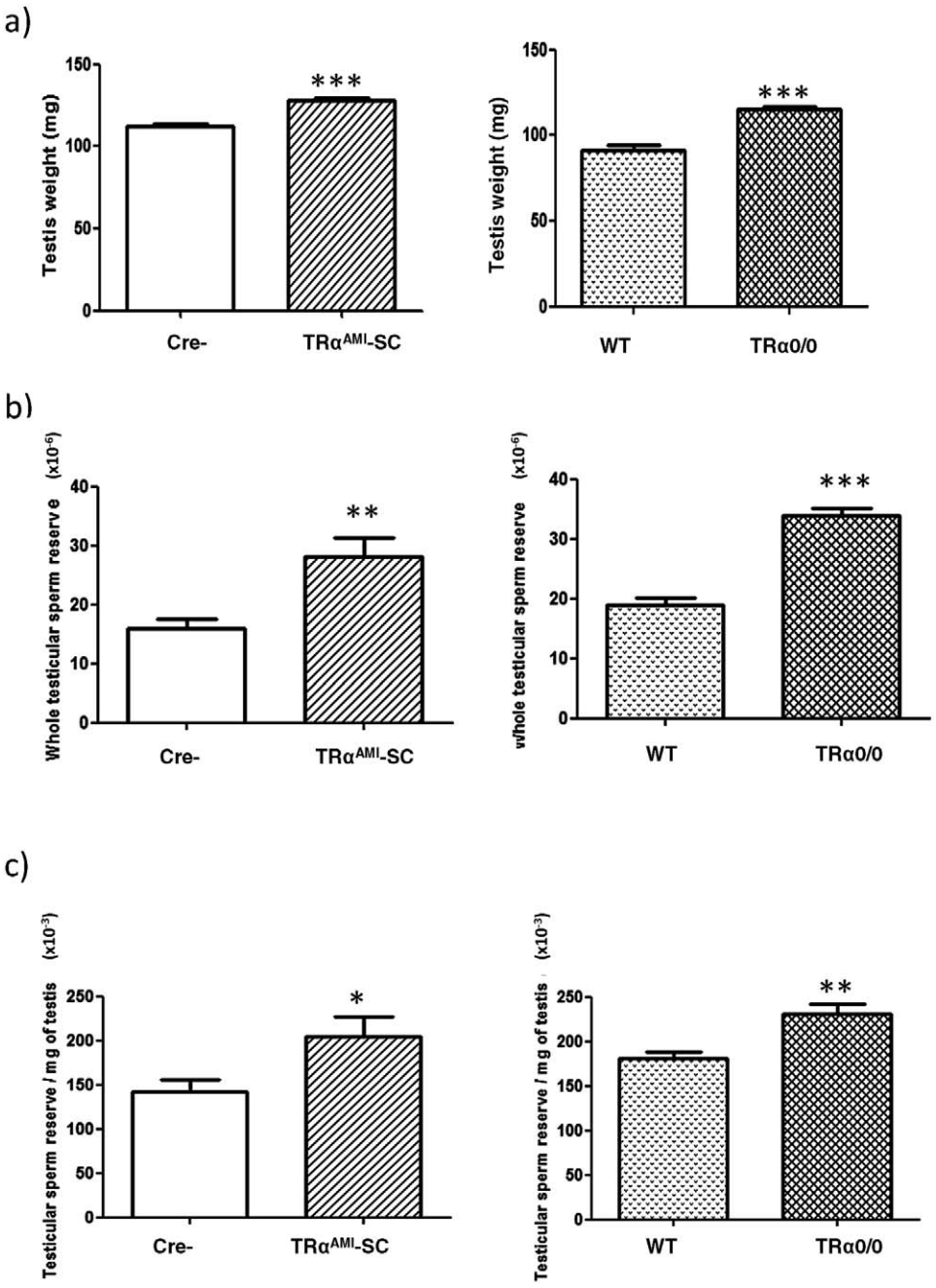

FIG. 1. Testis weight (**a**), whole testicular sperm reserve (**b**), and testicular sperm reserve per milligram of testis (**c**) in adult TRα^{AMI}-SC and TRα$^{0/0}$ mice (4 mo). A significant increase in testis weight (**a**) was observed in TRα^{AMI}-SC and TRα$^{0/0}$ mice in comparison with the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

within 3 h (Fig. 4a) was evaluated in TRα^{AMI}-SC at P0, P3, and P10, and in TRα$^{0/0}$ at P3. The SC proliferation index was significantly higher in TRα^{AMI}-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 TRα$^{0/0}$ 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 TRα^{AMI}-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 TRα$^{0/0}$ can be explained by the SC deficit in this line.

The Increase in SC Proliferation in TRα^{AMI}-SC and TRα$^{0/0}$ Mediated by TRα1 During Postnatal Development Is T3 Dependent

We wished to investigate if the control exerted by TRα1 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 TRα$^{0/0}$ and TRα^{AMI}-

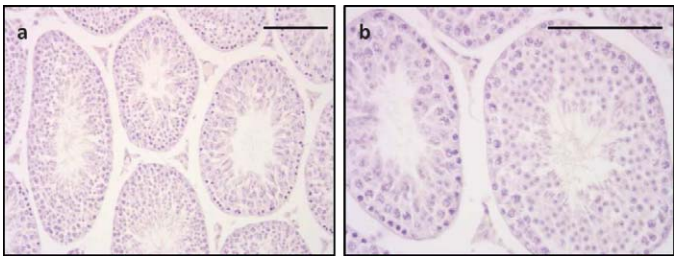

FIG. 2. Histological sections of adult TRα^{AMI}-SC testes at low (**a**) and high (**b**) magnifications (hematoxylin staining). Seminiferous tubules were fully and normally developed in TRα^{AMI}-SC, with their epithelium exhibiting normal structure and organization. Bar = 100 μm.

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 TRα^{AMI}-SC and Cre^{-} testes (Fig. 5a) or between TRα$^{0/0}$ and WT testes (Fig. 5b), indicating that the TRα1 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 TRα$^{0/0}$ and TRα^{AMI}-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 TRα^{AMI}-SC Testes at P3

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

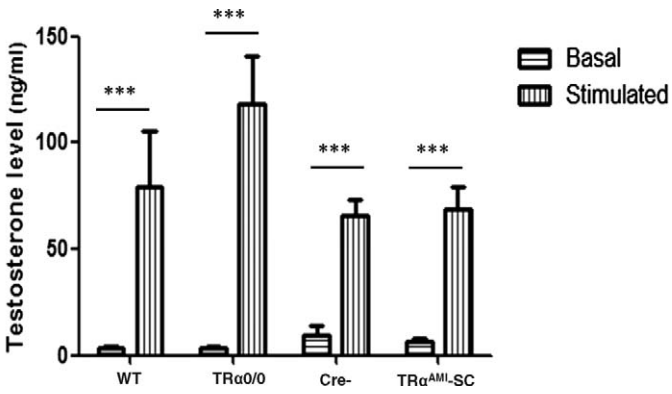

FIG. 3. Blood testosterone levels in TRα^{AMI}-SC and TRα$^{0/0}$ mice, in basal conditions and after hCG stimulation, compared to the respective control mice. No significant difference between the lines was observed for the basal and stimulated levels; hCG induced a significant increase (***$P < 0.001$)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

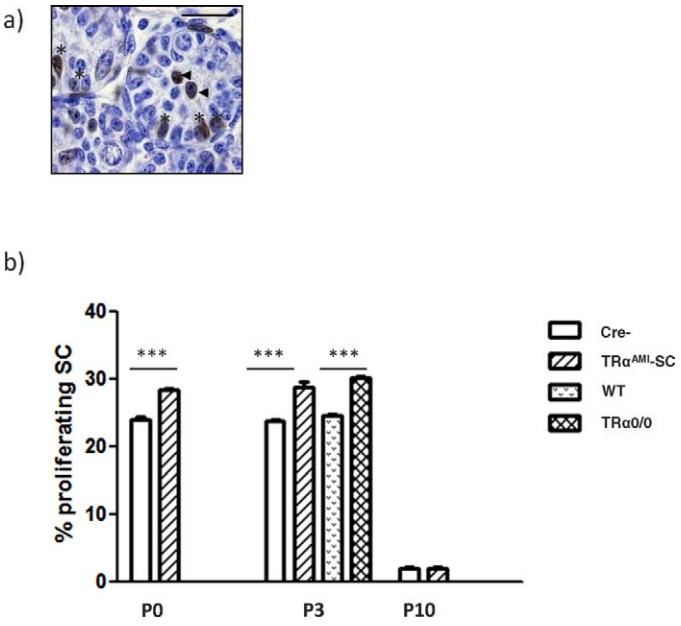

FIG. 4. Percentage of in vivo proliferating SCs in TRα^{AMI}-SC (at P0, P3, and P10) and in TRα$^{0/0}$ (at P3). **a**) Immunohistochemical labeling of proliferating SC (stars) and gonocytes (arrowheads) revealed by BrdU incorporation injected 3 h before death in P3 testes of TRα^{AMI}-SC. Bar = 20 μm. **b**) BrdU-negative and -positive SCs were counted and SC proliferation index was calculated. In TRα^{AMI}-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 TRα$^{0/0}$ 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 ± SEM; statistical analyses was by two-way ANOVA followed by Bonferroni post test.

TRα^{AMI}-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 TRα^{AMI}-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 TRα^{AMI}-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

TRα^{AMI}-SC and TRα$^{0/0}$ Exhibit the Same Testicular Phenotype

TRα^{AMI}-SC mice, where the dominant-negative TRα1^{L400R} mutation is expressed in SCs, display a testis phenotype that is

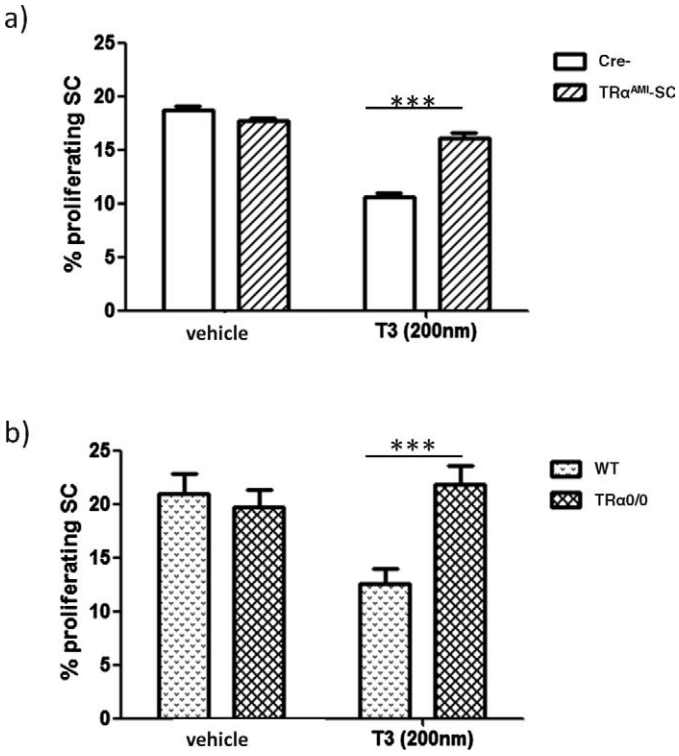

FIG. 5. SC proliferation index in testicular explants from TRα^{AMI}-SC and TRα$^{0/0}$ mice at P3, using organotypic cultures with or without exogenous T3. Organotypic cultures of P3 testes from TRα^{AMI}-SC (n = 6 for controls and n = 5 for TRα^{AMI}-SC [**a**]) and TRα$^{0/0}$ (n = 4 for WT and TRα$^{0/0}$ [**b**]) mice were performed with T3 (200 nM) or vehicle (PBS 1×, 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 TRα^{AMI}-SC and TRα$^{0/0}$ mice. Data are shown as the mean ± SEM; statistical analyses was by two-way ANOVA followed by Bonferroni post test.

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

To investigate further the TRα^{AMI}-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 TRα^{AMI}-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 TRα$^{0/0}$ 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 TRα^{AMI}-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 TRα^{AMI}-SC in comparison with the controls, as observed in TRα$^{0/0}$ 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 TRα^{AMI}-SC and TRα$^{0/0}$ mice.

In vivo, at P3, the SC proliferation index was significantly higher in both TRα^{AMI}-SC and TRα$^{0/0}$ 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 TRα1 impacts the proliferation rate rather than the length of the proliferative phase. This is corroborated by the fact that testicular TRα1 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 TRα1, 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 TRα^{AMI}-SC and TRα$^{0/0}$ is a strong indication that TRβ1 has no function in SC development, this possibility cannot be ruled out completely. First, TRβ1, 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 TRα$^{0/0}$ testis phenotype does not fully recapitulate postnatal hypothyroidism might be an indication that TRβ fulfills certain functions of some indirect T3 functions. In particular, TRβ could be acting in Leydig cells, maintaining testosterone levels. Second, if TRβ has a cell-autonomous function in SC during postnatal development, this might be inhibited by TRα1^{L400R}, as the two isoforms can cross-talk, at least in vitro. However, the systematic analysis of mice expressing TRα1^{L400R} in all tissues suggests that most TRβ functions are preserved in this model [25, 27].

Cell Cycle Actors Involved in SC Proliferation in TRα^{AMI}-SC and TRα$^{0/0}$

Gene expression profiling in testes at P3 highlighted changes in the expression of several genes in TRα^{AMI}-SC

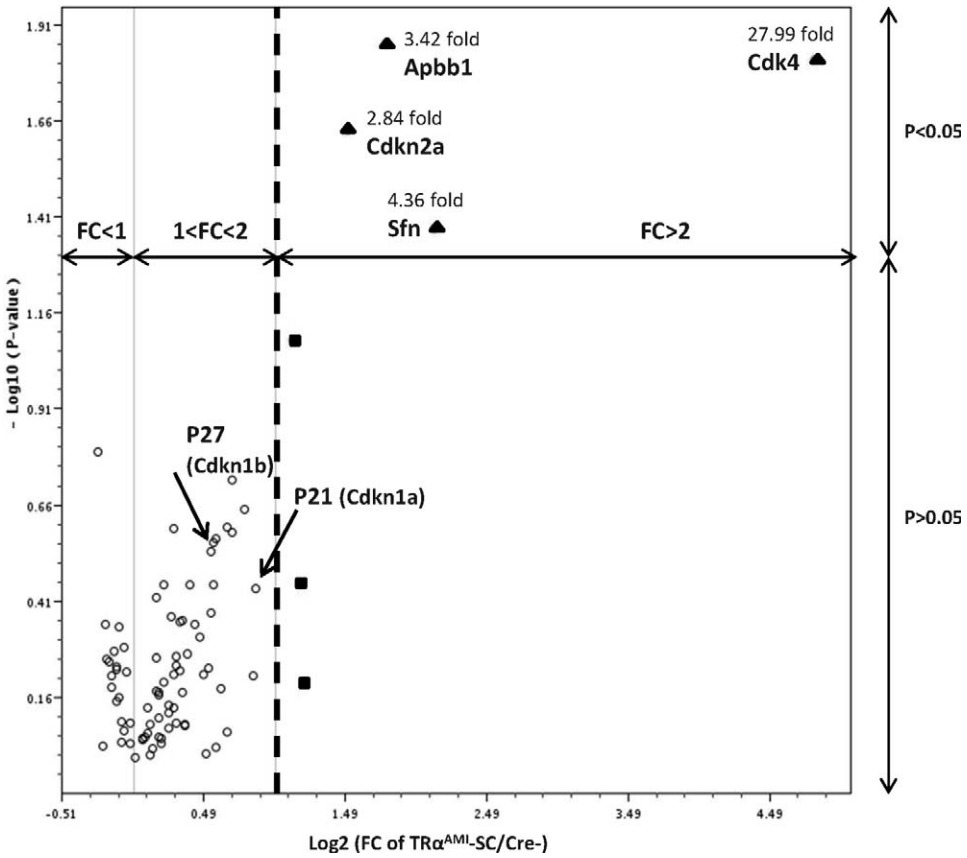

FIG. 6. Cell cycle gene expression is impacted in TRα^{AMI}-SC testes at P3. The "Volcano Plot" is an arbitrary representation (proposed by the Web-based RT^2 profiler PCR Array Data Analysis program) of the fold change (FC) for each of the 84 genes in the array. It represents the Log2 FC of each gene expression between the TRα^{AMI}-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 TRα^{AMI}-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 TRα$^{0/0}$ 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 TRα$^{0/0}$ [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 TRα1 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/μm^2), RS density (RSs/μm^2), and seminiferous tubule diameter in adult TRα^{AMI}-SC and Cre$^-$ (4 animals per genotype and 200 seminiferous tubules/animal).*

Parameter	Cre$^-$	TRα^{AMI}-SC	Mann-Whitney *U*-test	SAS combined process
SC/μm^2	$0.78\ 10^{-3} \pm 0.01\ 10^{-3}$	$0.96\ 10^{-3} \pm 0.02\ 10^{-3}$	$P < 0.001$	$P = 0.06$
RS/μm^2	$6.21\ 10^{-3} \pm 0.05\ 10^{-3}$	$5.90\ 10^{-3} \pm 0.08\ 10^{-3}$	$P = 0.11$	$P = 0.75$
Diameter (μm^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*).

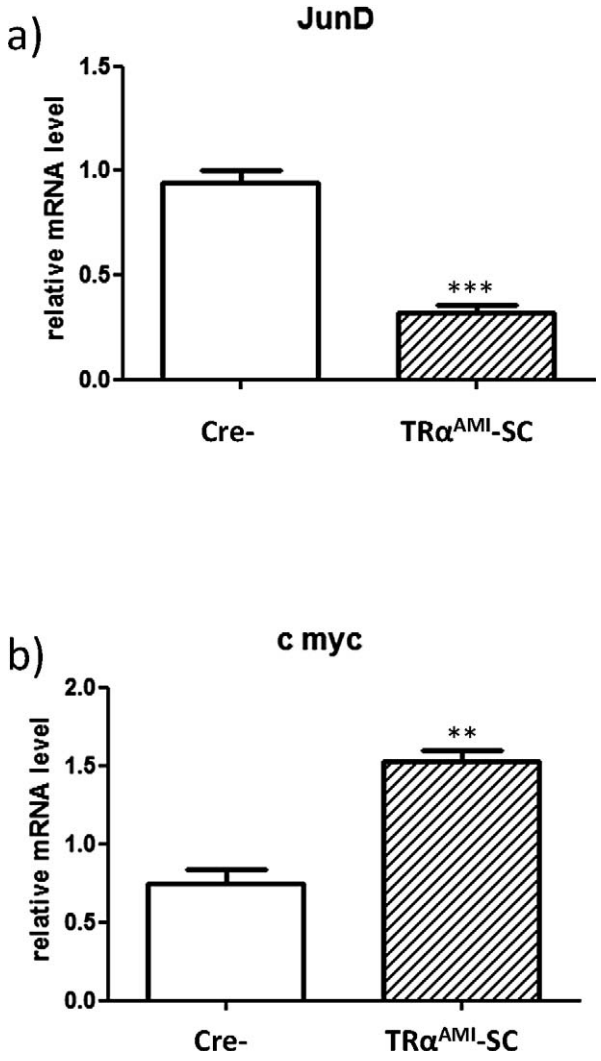

FIG. 7. JunD and c-myc gene expression is impacted in TRα^{AMI}-SC testes at P3. As JunD (**a**) and c-myc (**b**) were not present in the array, qRT-PCR analyses for these genes were performed with TRα^{AMI}-SC and control testes at P3 (n = 6 for each group) using specific primers (see Table 1). Normalization 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 ± SEM, and statistical analyses were performed using the Student *t* test. White bars, Cre^{-}; diagonal bars, TRα^{AMI}-SC.

The fact that TRα1 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 TRβ function, any antagonist compound displaying a marked preference for TRα1 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.

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