RESEARCH

A rise in T₃/T₄ ratio reduces the growth of prostate tumors in a murine model

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Abstract

Thyroxine (T₄) promotes cell proliferation and tumor growth in prostate cancer models, but it is unknown if the increase in the triiodothyronine (T₃)/T₄ ratio could attenuate prostate tumor development. We assessed T₃ effects on thyroid response, histology, proliferation, and apoptosis in the prostate of wild-type (WT) and TRAMP (transgenic adenocarcinoma of the mouse prostate) mice. Physiological doses of T₃ were administered in the drinking water (2.5, 5 and 15 µg/100 g body weight) for 6 weeks. None of the doses modified the body weight or serum levels of testosterone, but all of them reduced serum T₄ levels by 50%, and the highest dose increased the T₃/T₄ ratio in TRAMP. In WT, the highest dose of T₃ decreased cyclin D1 levels (immunohistochemistry) but did not modify prostate weight or alter the epithelial morphology. In TRAMP, this dose reduced tumor growth by antiproliferative mechanisms independent of apoptosis, but it did not modify the intraluminal or fibromuscular invasion of tumors. *In vitro*, in the LNCaP prostate cancer cell line, we found that both T₃ and T₄ increased the number of viable cells (Trypan blue assay), and only T₄ response was fully blocked in the presence of an integrin-binding inhibitor peptide (RGD, arginine-glycine-aspartate). In summary, our data show that the prostate was highly sensitive to physiological T₃ doses and suggest that *in vivo*, an increase in the T₃/T₄ ratio could be associated with the reduced weight of prostate tumors. Longitudinal studies are required to understand the role of thyroid hormones in prostate cancer progression.

Key Words

- ▶ prostate
- ▶ thyroid hormones
- ▶ cancer
- ▶ proliferation
- ▶ apoptosis
- ▶ TRAMP

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Introduction

Thyroid status controls prostate growth in critical periods of development. The continuous hypothyroidism or hyperthyroidism, both induced on day 30, decreased prostate weight in pubertal and adult rats (Maran *et al.* 1998, 2000). These studies show that serum levels of testosterone decrease in hypothyroidism and increase in hyperthyroidism, suggesting that testosterone is not

directly responsible for the prostatic weight reduction induced by the thyroid state (Maran *et al.* 1998, 2000). On the other hand, transient hypothyroidism (first 25 postnatal days) has a dual effect; it decreases prostate growth in puberty and induces prostate hypertrophy in adult rats (Cooke & Meisami 1991). This shift in the growth rate seems to be associated with delayed

puberty and extended cell proliferation (Wilson *et al.* 1997). Hence, these data demonstrate the relevance of maintaining a euthyroid state for the control of prostate growth.

Thyroid status regulates the activity of several metabolic enzymes, such as acid and alkaline phosphatases, Na^+/K^+ ATPase, Ca^{2+} ATPases, glycosidases and deiodinases in the prostate (Sidharthan *et al.* 1993, 1994, Maran *et al.* 1998, 2000, Anguiano *et al.* 2006). This regulation could be related to changes in the steroidogenic status, but also stimulatory effects of triiodothyronine (T_3) on glycosidase activity have been shown *in vitro* indicating a direct action of this hormone (Maran *et al.* 2000).

Studies in euthyroid rats show that 20% of prostatic T_3 content derives from thyroxine (T_4) deiodination (van Doorn *et al.* 1985), and that type 1 deiodinase (DIO1) enzyme is present since puberty in rat prostate (Anguiano *et al.* 2006). The DIO1 enzyme regulates the activation or inactivation of thyroid hormones (THs) (Luongo *et al.* 2019), and increments in DIO1 activity and local T_3 concentrations have been observed in the prostate of sexually active rats (López-Juárez *et al.* 2009, Delgado-Gonzalez *et al.* 2011). Besides deiodination, both mRNA and protein of nuclear thyroid receptors (TRs) have been identified in human prostate tissue (Sakurai *et al.* 1989), as well as in non-cancerous and cancerous prostate cell lines (Esquenet *et al.* 1995, Hsieh & Juang 2005). Thus, the presence of deiodinases/receptors make the prostate a target tissue for THs.

THs regulate numerous processes related to the hallmarks of cancer (Goemann *et al.* 2018, Krashin *et al.* 2019, Liu *et al.* 2019), but their role in prostate cancer has been controversial. In some studies, no relationship between thyroid function and the risk of developing cancer has been shown (Khan *et al.* 2016), but most of the works suggest or show that subclinical and overt hyperthyroidism (Tran *et al.* 2020), as well as high physiological levels of free T_4 and/or low levels of thyroid-stimulating hormone (TSH), increase the risk (Hellevik *et al.* 2009, Chan *et al.* 2017). In this context, hypothyroidism reduces or does not modify the risk of cancer in the prostate (Hellevik *et al.* 2009, Mondul *et al.* 2012). *In vitro*, T_4 stimulates the secretion of vascular endothelial growth factor (VEGF), and the migration and invasiveness of androgen-dependent (LNCaP) and independent (PC-3) prostate cancer cell lines (Delgado-González *et al.* 2016, Zhang *et al.* 2016). Besides, T_4 increases tumor growth in a PC-3 xenograft mouse model and hypothyroidism reduces it (Theodossiou *et al.* 1999). Thus, based on this evidence, T_4 seems to exert protumoral actions.

The role of T_3 in prostate cancer is less understood. T_3 at physiological concentrations (10 nM) stimulates cell proliferation in the LNCaP line when these cells are exposed to low concentrations (≤ 1 nM) of synthetic androgens, R1881 or mibolerone (Esquenet *et al.* 1995, Zhang *et al.* 1999). Also, it is known that T_3 does not modify cell proliferation in lines derived from normal epithelium or lines derived from androgen-independent prostate cancer, such as DU145 or PC-3 (Esquenet *et al.* 1995, Hsieh & Juang 2005). On the other hand, a physiological supplement of T_3 prevented cell invasion mediated by β -adrenergic activation in the LNCaP cell line and tumor growth in an LNCaP xenograft model (Delgado-González *et al.* 2016). These findings suggest that changes in the T_3/T_4 ratio could be relevant in the tumorigenic development of the prostate.

We hypothesized that physiological doses of T_3 would not affect the normal prostate of mice but could attenuate prostate cancer, based on the notion that an increase in the T_3/T_4 ratio could reduce tumor growth. The aim of this study was to analyze T_3 effects on the growth of the normal and cancerous prostate. We evaluated the histology, proliferation and apoptosis in the prostate of wild-type (WT) and TRAMP (transgenic adenocarcinoma of the mouse prostate) mice. T_3 and T_4 effects on cell viability were also analyzed *in vitro* in the LNCaP cell line.

Materials and methods

TRAMP model

All procedures were approved by the Bioethics Committee at Instituto de Neurobiología, Universidad Nacional Autónoma de México and followed the guidelines from the Institute of Laboratory Animal Resources Commission on Life Sciences National Research Council, USA. The TRAMP mice came from a C57/BL6 genetic background and were originally provided by Jackson Laboratories (Bar Harbor, ME, USA). TRAMP mice develop prostate intraepithelial neoplasia (PIN) and cancer at around 12 weeks of age, by the expression of PB-SV40-Tag early genes activated by the prostate-specific androgen-dependent promoter probasin (Gingrich *et al.* 1999). Tail DNA was used to identify the presence of the transgene by PCR. The primers used for amplification of product SV40 were designed by Jackson Laboratories (Table 1).

Cell line

Human prostate cancer cell line (LNCaP) is derived from a metastatic lymph node (CRL-1740) and was obtained

Table 1 Oligonucleotides used in genotyping and real-time PCR.

Target	Sequence (Mus musculus)	Amplicon length	GenBank
SV40	s CAGAGCAGAATTGTGGAGTGG as GGACAAACCACAAGTGAATGCAGTG	474	ID: 29031019
Trdc	s CAAATGTTGCTTGCTGGTG as GTCAGTCGAGTGCACAGTTT	200	ID: 100123473
Dio1	s ATTTGACCAAGTCAAGAGACTCGTAG as CCACGTTGTTCTTAAAAGCCCA	113	NM_007860.4
Dio2	s GGTGGTCAACTTTGGTTCAGCC as AAGTCAGCCACCGAGGAGAACT	96	NM_010050.3
Dio3	s TTCATGGCGCGGATGAG as GATGATAAGGAAGTCAACGTC	72	NM_172119.2
TRa1 or TRa1	s ACTACGTCAACCACCGCAAA as CCCTGAACAACATGCATTCCG	178	NM_001313983.1
TRb or TRb	s CCACTATCGCTGCATCACCT as TGTCTATGATGCACTTTCCTTCA	113	NM_001113417.1 NM_009380.3
Btg2	s AGCGAGCAGAGACTCAAGGT as GGGGTCCATCTTGTGGTTGA	138	NM_007570.2
Ccnd1	s GTGCAGAGGGAGATTGTGCC as GCGGCCAGGTTCCATTGAG	321	NM_007631.2
Gapdh	s GACAACTTTGGCATCGTGGA as ATGCAGGGATGATGTTCTGG	133	NM_001289726.1 NM_008084.3

Btg2, B-cell translocation gene 2; *Ccnd1*, cyclin D1; *Dio1*, type 1 deiodinase; *Dio2*, type 2 deiodinase; *Dio3*, type 3 deiodinase; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; *SV40*, Simian Virus 40 large T antigen; *Trdc*, T cell receptor delta, constant region (control gene); *TRa1*, thyroid receptor alpha1; *TRb*, thyroid receptor beta.

directly from ATCC (Lot number 62402565). LNCaP cell line was grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. Cultures were maintained at 37°C in a 5% CO₂ atmosphere and were used less than 15 passages.

Experimental designs

In vivo

WT and TRAMP mice at 8 weeks old were supplemented with 3,3',5-triiodo-L-thyronine sodium salt (T₃) (T6397, Sigma) in the drinking water at concentrations of 70, 150 and 450 µg/L for 6 weeks. Body weight (BW) was registered every week. Water consumption was normalized per day and divided between the number of mice per cage (3–4). The daily intake of T₃ was estimated by the amount of water ingested, and it was equivalent to 2.5, 5.0 and 15 µg T₃/100 g BW. Mice were euthanized at 14 weeks old. Serum was collected and prostate tissue was dissected, weighed, and stored in 10% formalin for histopathological and immunohistochemical analysis, or it was stored at –70°C for molecular and biochemical analysis. Serum levels of testosterone, T₄, T₃ and prostate concentrations of THs were measured by ELISA. TRs, deiodinases, a tumor suppressor (B-cell translocation gene 2, *Btg2*), cell proliferation regulators (cyclin D1 and proliferating cell nuclear antigen (PCNA)), and inducers of apoptosis (DNA fragmentation

factor and BAX protein levels) were analyzed by real-time PCR, immunohistochemistry and/or Western blot. The amount of tissue obtained from each lobe is a limitation in the mouse model. All analyses were performed in the whole prostate, except for the histopathological and immunohistochemical analyses, which were conducted only in the dorsolateral lobe. Three to four prostates were used to analyze each of the parameters.

In vitro

Before the addition of treatments, LNCaP cell line was cultured in RPMI-1640 medium supplemented with 10% charcoal-stripped FBS for 24 h. Cells were treated with a final physiological concentration of 10 nM free T₃ or 100 nM free T₄ (T2501, Sigma). Cell viability was respectively analyzed at 72 or 48 h of treatments by Trypan blue exclusion test. To analyze whether THs effects are mediated by αvβ3-integrin, LNCaP cell line was pre-incubated (2 h) and treated with a competitor peptide by the binding site to integrin (RGD, 500 µM) (A8052, Sigma) for 48 or 72 h. Every treatment was solubilized individually in RPMI charcoal-stripped FBS medium. *n* = 5–6 independent experiments by duplicate.

Histopathology

Prostate gland was dehydrated and embedded in paraffin, cut into 3-µm sections and stained with hematoxylin

and eosin. The analysis was performed on dorsolateral lobes of WT and TRAMP mice by light microscopy (Leica DM2500). Images were acquired with a Leica camera (DFC 420); and epithelial, lumen, and acinus sizes were analyzed with Leica Application Suite 4.9 at 10× magnification in three to four random fields and an average of 10 acini in WT and 15–20 acini in TRAMP. Pathological lesions were evaluated at 63× and categorized as prostate intraepithelial neoplasia (PIN) and well-differentiated cancer according to the proposed criteria (Berman-Booty *et al.* 2012). PIN was distinguished by the presence of epithelial cell stratification and short papillary projections protruding into the lumen and/or the presence of a cribriform pattern (intraductal invasion and fused epithelial niches). Cancer was mainly differentiated from PIN by the invasion of epithelial cells into the smooth muscle. All analyses were carried out in an average of five acini randomly taken at 10×. The lesions were confirmed by two observers.

Hormonal determinations in serum and prostate

Serum levels of testosterone (ALPCO, NH, USA), T₄ and T₃ (both from LSBio, WA, USA) were evaluated by mouse ELISA assays. For the quantification of prostatic THs content, the tissues were subjected to an extraction process in methanol/1 mM propylthiouracil. Samples were centrifuged at 800 *g* for 10 min, and the supernatant was removed and evaporated at 65°C. The percentage of recovery for both thyronines was 72% in both normal and cancerous tissues. The extracts were dissolved in human hypothyroid serum, and THs levels were quantified by human ELISA assay from International Immuno-Diagnostics (CA, USA).

Immunohistochemistry

Immunodetection for thyroid receptors TRα1 and TRβ, cyclin D1, and PCNA was carried out in paraffin sections. Antigen retrieval was performed by adding 0.05% citraconic anhydride solution pH 7.4 or 0.1 M citrate buffer pH 6.0 and heating in a pressure cooker for 30 min. Peroxidases were blocked with 3% hydrogen peroxide and incubated overnight at 4°C with the respective primary antibodies (Table 2). The sections were incubated at 37°C for 1 h with anti-rabbit IgG. Immunoreactivity was visualized with Vectastain ABC kit (Vector) using 3,3'-diaminobenzidine. Sections were counterstained with Gill's hematoxylin, dehydrated, and mounted in Entellan solution. Heart and liver were used as positive controls and testicle as a negative control for TRs. Prostate cancerous tissue was used as a positive control for PCNA and cyclin D1. Tissues incubated without primary antibodies were used as negative controls for the secondary antibody. Images at 63× were analyzed under light microscopy. Positive and negative cells per field were counted in an average of four fields randomly taken at 40×, using the cell counter plug-in from ImageJ 1.52a (NIH).

Immunoblotting

Cyclin D1, PCNA, BAX, and actin protein levels were determined by Western blot. Protein extracts equivalent to 25 or 50 µg were resolved through 12% SDS-PAGE and transferred to nitrocellulose membranes by electroblotting. Membranes were washed with Tris-buffered saline (TBS), pH 7.5 and blocked for 2 h with 5% non-fat milk in TBS+0.05% Tween 20 (TBST). Membranes were washed with TBST and incubated overnight at 4°C with their respective primary antibodies (Table 2).

Table 2 Primary antibodies.

Antigen	Supplier	Code	Origin	Working dilution
TRα1	Abcam	ab53729	Rabbit polyclonal	1:500/IHC
TRβ	Abcam	ab180612	Rabbit polyclonal	1:500/IHC
Cyclin D1	Santa Cruz	sc-450	Mouse protein fusion recombinant	1:100/IHC 1:500/WB
PCNA	Santa Cruz	sc-7907	Rabbit polyclonal	1:100/IHC 1:750/WB
BAX	Santa Cruz	sc 526	Rabbit polyclonal	1:1200/WB
Actin	Santa Cruz	sc 1616	Goat polyclonal	1:15,000/WB
Secondary antibodies				
Rabbit-IgG/Biotinylated	Vector	BA-1000	Goat polyclonal	1:1000/IHC
Mouse-IgG/Biotinylated	Vector	BA-2000	Horse polyclonal	1:1000/IHC
Rabbit-IgG/HRP	Santa Cruz	sc-2004	Goat polyclonal	1:5000/WB
Mouse-IgG/HRP	Santa Cruz	sc-2060	Goat polyclonal	1:5000/WB
Goat-IgG/HRP	Santa Cruz	sc 2020	Donkey polyclonal	1:5000/WB

Then, membranes were washed three times with TBST and incubated for 2 h with their respective secondary antibodies, anti-rabbit, anti-mouse and anti-goat conjugated to horseradish peroxidase (Table 2). Detection was performed using an enhanced chemiluminescence system. The membranes were analyzed by densitometry using ImageJ 1.52a. Data were normalized to the actin amount of each sample.

Real-time PCR

Gene expression was analyzed by real-time PCR. Total RNA was isolated from the whole prostate using Trizol reagent (Life Technologies). cDNA was synthesized with 2 μ g of total RNA using the M-MLV system. PCR was performed in the Rotor-Gene 3000 thermocycler (Corbett Research, Sydney, Australia) using a first denaturing step at 95°C for 4 min, followed by 35–45 cycles of three-step amplification (95°C, 30 s; 58–62°C, 30 s; 72°C, 30 s) and a final extension step at 72°C for 10 min. The primers are shown in Table 1. The melting curve and electrophoresis of the PCR product evidenced the presence of a single amplicon. No PCR products were observed in the absence of a template. Cycle thresholds (CT) of interest genes were normalized to a housekeeping reference gene (*Gapdh*) and with a control sample (untreated WT) by the $2^{-\Delta\Delta CT}$ method. Relative abundance of gene expression was reported as fold change regarding untreated WT sample, whose basal mean expression is 1.0.

DNA fragmentation

Prostate tissue was homogenized in ice-cold PBS in a 1:10 proportion and the apoptosis was evaluated by mouse DFFB sandwich ELISA, which measures the 40 kDa subunit of DNA fragmentation factor (LSBio). Data were normalized to the protein amount of each sample.

Statistical analysis

All data were plotted in a scatter plot to show the variability of the data. Data were analyzed by one- or two-way ANOVAs. The comparisons in the two-way ANOVA were performed between the six or eight experimental groups. Comparisons between groups were analyzed by post hoc Tukey. $P < 0.05$ was considered statistically significant, and differences were identified with letters. The lowest value from all groups was denoted with the letter 'a'. Analyses were performed using GraphPad Prism 6.

Results

T_3 did not modify the body weight or testosterone levels, but it reduced the weight of the cancerous prostate

None of the doses of T_3 modified the body weight gain or serum levels of testosterone in WT or TRAMP mice (Fig. 1A and B). A high variability in testosterone levels was found in both groups, but more homogenous levels were observed at the highest doses of T_3 in TRAMP. In both mice strains, all doses of T_3 decreased serum T_4 levels by 50%; and only the dose of 15 μ g/100 g BW increased serum levels of T_3 by 55% (Fig. 1C). TRAMP mice showed a prostatic weight higher than that of WT mice in basal conditions, but this increase was not observed with the highest dose of T_3 . This dose also decreased prostate weight in WT, but the reduction was not significant (Fig. 1D). The subsequent analyses were carried out with the lowest (2.5 μ g) and highest (15 μ g) doses of T_3 .

Figure 1

Body weight, serum hormone levels and prostate weight. (A) The final body weight was expressed as the percentage of change (%) regarding the initial weight. (B and C) Serum levels of T_4 and T_3 . (D) Wet prostate weight was normalized with respect to 100 g of body weight (BW). Mean of the data is shown in a scatter plot. Results were analyzed with a two-way ANOVA (dose/group), and differences between means were evaluated by the Tukey test. Different letters indicate significant differences $P < 0.05$. $n = 9$ mice per group for BW and prostate weight, and three to five mice per group for analysis of hormone levels.

T_3 regulated the expression of deiodinases and increased the prostatic T_3/T_4 ratio in TRAMP

No differences in prostatic T_4 or T_3 levels were observed between WT and TRAMP mice in basal conditions (Fig. 2A). The low dose of T_3 increased prostatic levels of T_4 only in TRAMP mice. In contrast, the high T_3 supplement induced a moderate (non-significant) decrease of this hormone in both groups. The high T_3 supplement increased prostatic T_3 concentrations in both mice strains and the T_3/T_4 ratio only in TRAMP.

Deiodinases expression was analyzed by real-time PCR (Fig. 2B). No significant differences in the basal expression of deiodinase type 1 (*Dio1*) or deiodinase 3 (*Dio3*) were observed between WT and TRAMP, but a low expression in deiodinase type 2 (*Dio2*) was observed in TRAMP compared to WT. Both doses of T_3 reduced *Dio1* and *Dio2* expression and increased *Dio3* expression in both mice groups.

Thyroid receptors (TRs) are present in normal and cancerous prostate

Gene expression of *TR α 1* and *TR β* was analyzed by real-time PCR (Fig. 3A) and protein levels by immunohistochemistry (Fig. 3B and C). No significant differences in the basal expression of *TR α 1* or *TR β* were observed between WT and TRAMP. Both doses of T_3 decreased *TR α 1* expression in both mice groups, but only the low dose increased *TR β* expression in WT (Fig. 3A). Figure 3B and C shows the immunolocalization of *TR α 1* and *TR β* . Both receptors are predominantly localized in the prostate epithelium. Nuclear and cytoplasm staining

was observed with both antibodies. There was no difference in *TR α 1* between groups at baseline, but a fewer *TR β* -positive cells were detected in TRAMP. The low dose of T_3 did not modify protein levels of receptors, but the high dose decreased *TR α 1* levels in TRAMP and increased *TR β* in WT. To analyze the relation between both subtypes, we found at baseline a low *TR β /TR α 1* ratio in TRAMP. The high T_3 dose increased this ratio two-fold in WT and four-fold in TRAMP.

T_3 reduced the epithelium area and acinus size in TRAMP, but not in WT

Figure 4A shows a representative image of prostate histology. TRAMP mice showed an increased epithelial area and higher epithelium/lumen ratio than those of WT mice (Fig. 4B). T_3 did not modify the epithelial area in WT mice, but a reduction in the luminal area and acini size was observed. Both doses of T_3 reduced the epithelial area in TRAMP, and only the high dose reduced the acini size. T_3 had no effects on the epithelium/lumen ratio.

T_3 did not modify prostate cancer progression

Figure 5A shows a representative image of a stratified proliferative niche (black arrow) and a cribriform pattern (white arrow) characteristic of PIN; as well as the loss of basal lamina and the epithelial invasion of smooth muscle (fibromuscular invasion) distinctive of cancer (gray arrow). Figure 5B, C and D shows that T_3 intake did not significantly modify the incidence of PIN or cancer nor the number of cribriform structures per acinus or the number of acini with fibromuscular invasion.

Figure 2

Prostatic levels of THs and deiodinase expression. (A) Prostatic concentrations of T_4 and T_3 were measured by ELISA. (B) Deiodinase mRNA levels were measured by real-time PCR. Mean of the data is shown in a scatter plot. Results were analyzed with a two-way ANOVA (dose/group), and differences between means were evaluated by the Tukey test. Different letters indicate significant differences $P < 0.05$. $n = 3$ mice/group.

Figure 3

Expression and immunolocalization of thyroid hormone receptors. (A) mRNA levels of $TR\alpha1$ and $TR\beta$ were determined by real-time PCR. (B) Representative images of immunohistochemistry for $TR\alpha1$ and $TR\beta$ in dorsolateral lobe. Calibration bar, 20 μm for 63X magnification. (C) $TR\alpha1$ and $TR\beta$ -positive cells were quantified and normalized regarding total cells. Mean of the data is shown in a scatter plot. Results were analyzed with a two-way ANOVA (dose/group), and differences between means were evaluated by the Tukey test. Different letters indicate significant differences $P < 0.05$. $n = 3-4$ mice/group. A full color version of this figure is available at <https://doi.org/10.1530/JOE-20-0310>.

T_3 decreased PCNA levels in both mice groups but reduced cyclin D1 only in WT

No significant differences in basal gene expression of *Btg2* or *Ccnd1* (cyclin D1) were observed between WT and TRAMP. The high T_3 dose decreased the expression of *Ccnd1* in both groups, and *Btg2* reduction was significant only in TRAMP (Fig. 6A). No differences in cyclin D1 levels were observed between WT and TRAMP (Western blot or immunohistochemistry) (Fig. 6B and C). However, the opposite effects were observed in the T_3 -treated groups. In WT mice, T_3 did not modify total protein levels of cyclin D1 but reduced the number of positive cells. In TRAMP, the low T_3 dose increased the total protein levels of cyclin D1 but not the number of positive cells.

There was no difference in the basal levels of PCNA protein when it was analyzed by Western blot or immunohistochemistry (Fig. 7A and B). The low T_3 dose reduced total protein levels of PCNA but not the number of positive cells in WT mice, whereas the high dose of T_3 increased the total levels of PCNA but reduced the number of PCNA-positive cells in TRAMP.

T_3 did not induce apoptosis

Apoptosis was analyzed through DNA fragmentation and determination of BAX protein levels. No significant differences of apoptosis (DFFB assay) were observed between WT and TRAMP in basal conditions or in response to T_3 , but the low dose of T_3 selectively incremented BAX protein levels in WT mice (Fig. 8A and B).

T_3 and T_4 increased cell proliferation in the LNCaP line, but only $\alpha v\beta3$ integrin mediated the effects of T_4

Physiological concentrations of T_3 and T_4 increased around 30–35% the number of cells of the LNCaP cell line at 72 and 48 h of incubation, respectively. RGD peptide fully blocked the increment associated with T_4 and partially T_3 (Fig. 9A and B).

Discussion

We examined the physiological effects of a T_3 supplement on normal and cancerous prostate. A crucial finding was

Figure 4

Histological analysis of prostate in WT and TRAMP mice. (A) Representative images of slices stained with hematoxylin-eosin captured at 10X magnification. Calibration bar, 200 µm. (B) Quantitative analysis of epithelial and luminal area, and acinus size. Mean of the data are shown in a scatter plot. Results were analyzed with a two-way ANOVA (dose/group), and differences between means were evaluated by the Tukey test. Different letters indicate significant differences $P < 0.05$. $n = 4$ mice/group. A full color version of this figure is available at <https://doi.org/10.1530/JOE-20-0310>.

that T_3 does not affect the weight or cytoarchitecture of the normal prostate; but in tumors, it increases the T_3/T_4 ratio and reduces its growth probably by antiproliferative mechanisms.

As expected, all doses of T_3 decreased T_4 levels in serum by a negative feedback mechanism, and only the highest dose (15 µg/100 g BW) increased serum T_3 levels by 40%. This, and the lack of effects of T_3 on the BW suggest that these doses were within a physiological range. Deiodinases and TRs are two important elements in T_3 signaling. The availability of T_3 is regulated by type 1 (DIO1), type 2 (DIO2), and type 3 (DIO3) enzymes. DIO1 catalyzes either 5' or 5-deiodination and promotes the generation of T_3 or inactivation of T_4 and T_3 , whereas DIO2 and DIO3 catalyze 5' and 5-deiodination, respectively (Luongo *et al.* 2019). Deiodinase regulation has been studied in different types of neoplasias, and depending on the type and stage of cancer, DIO1 and DIO2 are up or downregulated, whereas DIO3 is usually

induced (Goemann *et al.* 2018). Deiodinases have not been previously studied in mouse prostate. Rat prostate expresses mRNA for both *Dio1* and *Dio2*, but only DIO1 activity was identified in the normal gland during puberty (Anguiano *et al.* 2006). The cycle threshold (CT) values of the real-time PCR showed overexpression of *Dio1* (100 times) and *Dio2* (200 times) with regard to *Dio3* in both groups of untreated mice (data not shown), suggesting the prevalence of the activation pathway (*Dio1* or *Dio2*) in basal conditions. An important finding was that even the low dose of T_3 activates the mechanisms aimed at maintaining a euthyroid prostatic environment by inhibiting *Dio1* and *Dio2* and inducing *Dio3*. This data is consistent with the fact that T_3 is a central regulator in the expression of deiodinases in multiple tissues (St. Germain *et al.* 1994). Interestingly, the two- to three-fold inhibition in *Dio1* and *Dio2* and the robust induction in *Dio3* did not induce local hypothyroidism, indicating that other mechanisms such as activation of the DIO2 enzyme

Figure 5

Pathological analysis of dorsolateral lobes in TRAMP mice prostate. (A) Representative images of prostate lesions captured at 40X magnification (hematoxylin–eosin stain). The black arrow shows a hyperplastic niche; the white arrow shows the presence of a cribriform structure; and the grey arrow indicates the site of invasion of epithelial cells into fibromuscular tissue. (B) Percentage of acini with normal phenotype, intraepithelial hyperplasia (PIN) and cribriform pattern (cancer). Bars represent mean $\pm$ s.d. (C) Incidence of cribriform structures per acinus. (D) Percentage of acini that present fibromuscular invasion. Mean of the data are shown in a scatter plot. Results were analyzed with a one-way ANOVA (dose/group). No significant differences were founded. $n = 3\text{--}4$ mice/group. A full color version of this figure is available at <https://doi.org/10.1530/JOE-20-0310>.

and/or membrane transporters also participate in prostatic availability of T_3 .

In the prostate of TRAMP mice, but not in WT, the high dose of T_3 increased the T_3/T_4 ratio, suggesting that thyroid equilibrium is compromised in cancer. Unlike other tissues (i.e. liver, kidney, thyroid), a direct increase in *Dio1* expression by T_3 was not observed here. This could be explained by the non-hyperthyroid doses of T_3 and/or by organ-specific mechanisms. DIO1 enzymatic activity is differentially regulated in liver and prostate by prolactin, 17 β -estradiol and testosterone in rats (Anguiano *et al.* 2006). The increment in prostatic T_4 concentrations in TRAMP mice only at low T_3 doses might be explained by

a possible differential response of the T_4 transporters to T_3 concentrations. More studies are needed to understand the contribution of transports/deiodinases to thyroid homeostasis in the prostate.

TH signaling involves the activation of canonic (DNA binding nuclear receptors) and non-canonical pathways (Vella & Hollenberg 2017, Davis *et al.* 2018, Liu *et al.* 2019). For the first time, this study shows the presence of TRs and their regulation by T_3 in the normal and cancerous prostate. In basal conditions, the values of CT indicate that *TR α 1* expression is 1000 and 100-fold more expressed than *TR β* in WT and TRAMP mice (data not shown). These data agree with the high *TR α* expression reported in benign tumor prostate (Sakurai *et al.* 1989). Both doses of T_3 markedly decreased *TR α 1* expression in normal and cancerous prostate. Indirect mechanisms are surely involved because the *TR α 1* gene is not directly regulated by thyroid response elements (TREs) (Ishida *et al.* 1993, Baas *et al.* 1998). In contrast, T_3 strongly stimulated *TR β* expression, probably by a direct mechanism via TREs (Sakurai *et al.* 1992). *TR β* protein was previously detected in normal and prostate cancer cell lines (Hsieh & Juang 2005). Here, we showed that there were no direct associations between mRNA and protein levels (except for the high dose of T_3 in WT mice), supporting the notion of complex transcriptional and/or post-translational regulation (Yen 2001). Our data showed a 55 and 200% more positive cells for *TR α 1* than for *TR β* in WT and TRAMP mice, respectively. However, a switch occurs in response to T_3 , increasing the *TR β /TR α 1* ratio in both mice groups. During development, both subtypes can regulate cell proliferation, survival and cell differentiation in an organ-specific manner (White *et al.* 2001), and it is known that reactivation of the T_3 /*TR β* axis induces a differentiation program in hepatic cancer (Kowalik *et al.* 2020). Additional studies are required to understand the functional significance of these receptors in the prostate gland.

A major finding was that the increment in the T_3/T_4 ratio reduced tumor growth in TRAMP, as evidenced by a reduction in the total prostate weight and a diminution in the epithelial area, acinus size and number of PCNA-positive cells in the dorsolateral lobe. PCNA participates in the replication and repair of DNA and has been widely used as a cell proliferation marker (Naryzhny 2008). Although T_3 did not reduce PCNA levels in the whole prostate (Western blot), the reduction of PCNA in the dorsolateral lobe suggests that the antiproliferative effects of T_3 could be lobe specific. Rodent prostate is composed of ventral, dorsolateral and anterior lobes; and although

Figure 6

Cell proliferation mediators. (A) mRNA levels of *Btg2* and *Ccnd1* (Cyclin D1) were measured by real-time PCR. (B) Western blot and densitometric analysis for Cyclin D1. (C) Immunohistochemistry and analysis of Cyclin D1-positive cells.

Representative images were captured at 63X magnification. Calibration bar, 20 μm . Mean of the data is shown in a scatter plot. Results were analyzed with a two-way ANOVA (group/dose), and differences between means were evaluated by the Tukey test. Different letters indicate significant differences $P < 0.05$. $n = 3-4$ mice/group. A full color version of this figure is available at <https://doi.org/10.1530/JOE-20-0310>.

all lobes develop cancer in the TRAMP model (Berman-Booty *et al.* 2012), the dorsolateral lobe is the most widely studied in cancer because of its molecular homology (mRNA signature) to the peripheral zone of human prostate, which is highly prone to developing neoplasia (Berquin *et al.* 2005).

In certain cancers, T_3 negatively regulates *Ccnd1* and *Btg2* gene expression (Garcia-Silva *et al.* 2002, Natsume *et al.* 2003, Michienzi *et al.* 2007). *Ccnd1* codes for cyclin D1 an activator and regulator of cell cycle (Tashiro *et al.* 2007), while *Btg2* codes for a protein involved in cell arrest and apoptosis (Yuniati *et al.* 2019). T_3 reduced cyclin D1 expression in both mice groups; but cyclin D1 protein only was reduced in WT, suggesting that antiproliferative T_3 effects are cyclin D1-independent in TRAMP. In this transgenic context, T_3 unresponsiveness of cyclin D1 could be inherent to tumor antigens (*SV40*) and to the constitutive activation of this cyclin (Ahuja *et al.* 2005). Other cyclins, such as E2, A2, and B1 participate in the transition to cancer (Maddison *et al.* 2004), and its regulation by T_3 should be analyzed. In the LNCaP cell line, it has shown that proliferative T_3 effects involve *Btg2* downregulation (Tsui *et al.* 2008), and although in our study this inhibition was confirmed, no hyperplasia or prostate hypertrophy were observed, suggesting the activation of counterregulatory mechanisms. Indeed, it has shown that T_3 reduces tumor growth in an LNCaP xenograft model

(Delgado-González *et al.* 2016). Pro- and antiproliferative T_3 effects are not easy to understand, but intrinsic elements to the *in vitro* and *in vivo* conditions could mediate these differences. On the other hand, evidence shows that T_3 does not alter cell proliferation in a normal prostate cell line (Hsieh & Juang 2005), consistently we found that T_3 effects in cyclin D1 and Bax were not accompanied by epithelial alterations in WT mice.

Proliferative effects of THs are also mediated by $\alpha v \beta 3$ integrin; whose pathway is activated with physiological or supraphysiological concentrations of T_4 and T_3 , respectively, and involves the activation of the mitogen-activated protein kinase (MAPK) (Bergh *et al.* 2005, Scarlett *et al.* 2008, Davis *et al.* 2018). Both THs increased the proliferation in the LNCaP cell line, and although only T_4 response was $\alpha v \beta 3$ integrin-dependent, our study does not rule out that T_3 effects could be mediated by integrin (RGD-partial inhibition). Evidence shows that $\alpha v \beta 3$ integrin is incremented in prostate tumors and prostate cancer cell lines (Goel *et al.* 2008), and that T_4 increases VEGF secretion and invasive potential in the LNCaP and/or PC-3 cell lines (Delgado-González *et al.* 2016, Zhang *et al.* 2016). More detailed analyses of the integrin-dependent and -independent mechanisms are required.

On the other hand, our work shows that apoptosis is not the main mechanism involved in the reduction

Figure 7

Protein levels and immunolocalization for PCNA. (A) Representative images of Western blot and densitometric analysis. (B) Representative images of immunohistochemistry and analysis of PCNA-positive cells. Calibration bar, 20 μm at 63X magnification. Mean of the data is shown in a scatter. Results were analyzed with a two-way ANOVA (group/dose), and differences between means were evaluated by the Tukey test. Different letters indicate significant differences $P < 0.05$. $n = 3-4$ mice/group. A full color version of this figure is available at <https://doi.org/10.1530/JOE-20-0310>.

Figure 8

Apoptosis analysis. (A) DFFB assay measures DNA fragmentation. (B) Representative images of Western blot and densitometric analysis for BAX protein. Mean of the data is shown in a scatter plot. Results were analyzed with a two-way ANOVA (group/dose). No significant differences were found. $n = 3-4$ mice/group.

of prostate tumors. A recent study shows that T_3 induces cell arrest by senescence in prostate cancer cell lines (Kotollosi *et al.* 2020). Interestingly, *in vivo* T_3 reduced tumors weight; but did not attenuate nor magnified cancer progression. However, a longitudinal analysis must perform to understand THs effects on prostate cancer. There is evidence that low levels of TSH and high levels of free T_4 could be a factor that predicts the risk of developing prostate cancer (Chan *et al.* 2017).

It is unlikely that THs effects are related with metabolism or gonadal activity, since neither the BW of mice nor testosterone levels were modified. Androgens are crucial on prostate pathophysiology; and although their interrelationship with T_3 has been poorly analyzed, it is known that *in vitro* T_3 increases androgen receptor levels (Esquenet *et al.* 1995); and androgen deprivation

Figure 9

Effects of THs on the cell viability of the LNCaP cell line. (A and B) Cells were incubated with T_3 and T_4 for 72 and 48 h, respectively. RGD was preincubated for 2 h and remained throughout the study. Mean of the data is shown in a scatter plot. Data represent percentage of change regarding the initial number of cells (time 0). Results were analyzed with a one-way ANOVA, and differences between means were evaluated by the Tukey test $P < 0.05$. $n = 5$ –6 independent experiments per duplicate.

decreases the T_3/T_4 ratio in the serum of prostate cancer patients (Hoermann *et al.* 2017).

In summary, our working hypothesis was partially confirmed. We hypothesized that physiological doses of T_3 would not affect the normal prostate, but the increase in the T_3/T_4 ratio could attenuate prostate cancer. Our data showed that both the normal and cancerous prostates were sensitive to physiological doses of T_3 and revealed the importance of maintaining the concentrations of T_3 to keep the integrity of prostate epithelium or increasing the T_3/T_4 ratio to counteract the growth of cancerous prostate. However, a detailed analysis per lobes and a time course of tumor development deserve further investigation.

Declaration of interest

The authors declare that there is no conflict of interest could be perceived as prejudicing the impartiality of the research reported.

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