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Mice deficient in the steroid receptor co-activator 1 (SRC-1) are resistant to thyroid hormone

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Steroid receptor co-activator 1 (SRC-1) is a transcription co-factor that enhances the hormone-dependent action, mediated by the thyroid hormone (TH) receptor (TR) and other nuclear receptors. *In vitro* studies have shown that SRC-1 is necessary for the full expression of TH effect. SRC-1 knockout mice (SRC-1$^{-/-}$) provide a model to examine the role of this co-activator on TH action *in vivo*. At baseline, SRC-1$^{-/-}$ mice display resistance to TH (RTH) as evidenced by a 2.5-fold elevation of serum TSH levels, despite a 50% increase in serum free TH levels as compared with wild-type (SRC-1$^{+/+}$) mice. When mice were made hypothyroid, TSH levels increased, obliterating the difference between SRC-1$^{+/+}$ and SRC-1$^{-/-}$ mice observed at baseline. In contrast, the decline of TSH by treatment with L-triiodothyronine was severely blunted in SRC-1$^{-/-}$ mice. These data indicate that SRC-1 is not required for the upregulation of TSH in TH deficiency. However, SRC-1 enhances the sensitivity of TSH downregulation by TH. This is the first demonstration of RTH caused by a deficient co-factor other than TR. It supports the hypothesis that a putative defect in the SRC-1 gene or another co-factor could be the cause of RTH in humans without mutations in the TR genes.

Keywords: co-activator/resistance to thyroid hormone/SRC-1/thyroid hormone receptor/thyrotropin

Introduction

Thyroid hormone (TH) action is mediated through specific TH receptors (TRs) functioning as ligand-dependent transcription factors that increase or decrease the expression of target genes, depending on whether the gene promoters contain, respectively, positively or negatively regulated TH response elements (Yen and Chin, 1994; Mangelsdorf *et al*., 1995). On positive TH response elements, the unliganded TRs suppress basal gene transcription activity by interacting with co-repressors such as nuclear co-repressor (NCoR), silencing mediator of retinoic acid (SMRT) and triiodothyronine (T_3) receptor-associating co-factor (TRAC) (Chen and Evans, 1995; Horlein *et al*., 1995; Kurokawa *et al*., 1995; Sande and Privalsky, 1996), associated with histone deacetylase via an intermediate factor (Nagy *et al*., 1997). Conformational changes of the TR, produced by T_3 binding, release the co-repressor complex and recruit co-activators such as steroid receptor co-activator 1 (SRC-1), transcriptional intermediary factor 2 (TIF2) and p300/cAMP response element-binding protein-interacting protein (p/CIP) (Onate *et al*., 1995; Voegel *et al*., 1996; Hayashi *et al*., 1997; Torchia *et al*., 1997), all exhibiting histone acetyltransferase activity. The latter modifies the structure of the chromatin and, through loosening the nucleosome at the site of the promoter, activates gene transcription (Adams and Workman, 1993; Wolffe and Pruss, 1996). *In vitro* experiments show that promoter sequences of genes regulated negatively by TH are stimulated by the unliganded TR and repressed with the addition of TH, by mechanisms that are not well understood (Hollenberg *et al*., 1995; Tagami *et al*., 1997). These experiments serve as prototypes for the *in vivo* effects of TH on thyrotropin (TSH) gene expression.

Resistance to thyroid hormone (RTH) is an inherited syndrome of variable tissue hyposensitivity to thyroid hormone (Refetoff and Weiss, 1997). The diagnostic features of the syndrome are elevated free TH concentrations in serum without the expected suppression of TSH. RTH is caused by mutations in the *TR*β gene that have been identified in affected subjects belonging to >150 families (Announcement, 1994; unpublished observation). Most commonly, mutations are located in the ligand-binding domain of the TRβ, which reduce its affinity for TH and interfere with the function of the wild-type TR to produce dominantly inherited RTH (Adams *et al*., 1994; Hayashi *et al*., 1995). Recently, some mutant TRβs were found to have impaired interaction with one of the co-factors involved in the regulation of TH action (Yoh *et al*., 1997; Collingwood *et al*., 1998; Liu *et al*., 1998; Tagami *et al*., 1998). Pertinent to the current work are mutant TRβs with reduced ligand-dependent transactivation that appears to be due in part to a weaker interaction with the co-activator, SRC-1 (Collingwood *et al*., 1998; Liu *et al*., 1998). The identification of RTH in the absence of mutations in the TRβ or TRα genes (Weiss *et al*., 1996) lends further support to the hypothesis that defective co-factors could, by themselves, cause RTH.

In vitro studies have shown that SRC-1, first identified as steroid receptor co-activator (Onate *et al*., 1995), functions as a co-activator of other nuclear receptors, including the TR (Jeyakumar *et al*., 1997; Feng *et al*., 1998). Also known as the nuclear co-activator 1 (NcoA-1), SRC-1 is widely distributed in tissues that express TRs, including the pituitary gland (Misti *et al*., 1998). The opportunity to establish the physiological role of SRC-1 in the mediation of TH action and to determine whether or not this ubiquitous co-activator may be involved in the

Table I. Thyroid function tests in SRC-$1^{+/+}$ and SRC-$1^{-/-}$ mice

Genotype[a]	Total T_4 (μg/dl)	FT_4I	Total T_3 (ng/dl)	FT_3I	TSH (ng/ml)
SRC-$1^{+/+}$					
Male (27)	3.51 ± 0.61	5.51 ± 1.53	80.1 ± 14.7	108.6 ± 35.8	0.17 ± 0.17
Female (15)	3.44 ± 0.68	6.35 ± 1.49	72.9 ± 9.9	131.8 ± 13.8	0.09 ± 0.02
Combined (42)	3.48 ± 0.63	5.78 ± 1.27	77.5 ± 13.5	125.7 ± 23.1	0.13 ± 0.13
SRC-$1^{-/-}$					
Male (27)	4.79 ± 0.83***	8.59 ± 1.53***	126.0 ± 25.7***	259.8 ± 42.0***	0.52 ± 0.61*
Female (12)	5.49 ± 0.89***	9.09 ± 1.83***	113.0 ± 15.6***	187.4 ± 20.8***	0.42 ± 0.67
Combined (39)	5.00 ± 0.89***	8.73 ± 1.61***	122.1 ± 23.7***	211.5 ± 45.1***	0.48 ± 0.63**

[a]Number of mice used indicated in parentheses.
Difference between SRC-$1^{+/+}$ and SRC-$1^{-/-}$ mice: *p <0.05; **p <0.01; ***p <0.001.

syndrome of RTH arose with the development of mice deficient in SRC-1 (SRC-$1^{-/-}$) (Xu *et al*., 1998). Though fertile, these mice exhibit partial resistance to sex steriod hormones as evidenced by decreased growth and development of target tissues and reduced responses to estrogen and androgen.

Our studies show that the SRC$^{-/-}$ mice exhibit the cardinal features of RTH in humans, namely increased serum free TH [thyroxine (T_4) and T_3] levels, associated with high concentration of TSH. Furthermore, compared with the wild-type (SRC-$1^{+/+}$) mice, larger amounts of L-T_3 were required to suppress their serum TSH. In contrast, upregulation of TSH by TH deprivation was not impaired in the SRC$^{-/-}$ mouse.

The SRC-1 knock-out mouse provides a model for the detailed investigation of the regulation of TH action in the absence of one specific co-activator, SRC-1, through hormonal manipulations that could not be carried out in humans.

Results

Parameters of thyroid function at baseline in adult SRC-$1^{-/-}$ mice as compared with SRC-$1^{+/+}$ mice

Results of thyroid function tests in untreated mice of both types are shown in Table I. Both female and male SRC-$1^{-/-}$ mice manifested the characteristic hormonal changes associated with RTH. Serum total and free T_4 and T_3 levels, as well as TSH concentrations were significantly higher in SRC-$1^{-/-}$ as compared with SRC-$1^{+/+}$ mice. T_4 and T_3 concentrations in the SRC-$1^{-/-}$ mice were ~0.5-fold above and TSH concentrations were 2.5-fold above those in SRC-$1^{+/+}$ mice. Similar differences were observed in subsequent experiments at baseline (Figures 1 and 2). Because of age and sex differences in TH levels, and in particular, marked sex differences in serum TSH concentrations in intact wild-type mice, subsequent experiments were performed in adult male mice because their higher baseline TSH levels allow to quantitate the suppressive effect of L-T_3 administration.

The role of SRC-1 on TSH regulation in the absence of TH

To determine the role of SRC-1 on a TH-regulated gene in the absence of TH, we studied the upregulation of TSH during the course of TH deprivation induced by treatment with low iodine diet containing propylthiouracil (Lo I/PTU) (Figure 1). The difference in TSH concentration between SRC-$1^{-/-}$ and SRC-$1^{+/+}$ mice was obliterated

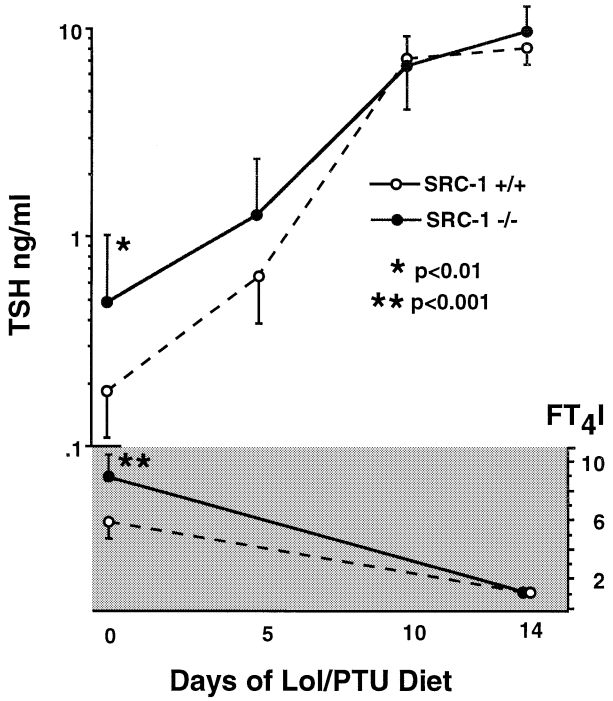

Fig. 1. Effect of TH-deprivation on serum TSH concentrations (upper panel) and FT_4I levels (shaded panel). Determinations were performed on serum samples obtained at baseline, 5, 10 and 14 days after beginning of Lo I/PTU diet. Asterisks identify p values for differences between SRC-$1^{+/+}$ and SRC-$1^{-/-}$ mice. Each point is the mean ± SD for 12 mice of each type except for the last two points (14 days) which represent 5 and 6 SRC-$1^{+/+}$ and SRC-$1^{-/-}$ mice, respectively.

by day 10 of Lo I/PTU diet and remained so on the fourteenth day, at which time mean concentrations were 9.8 ± 1.8 and 8.1 ± 1.4 ng/ml, respectively. Fourteen days of Lo I/PTU diet produced a decline in serum free T_4 index (FT_4I) to the same level in both SRC-$1^{-/-}$ and SRC-$1^{+/+}$ mice of 0.66 ± 0.11 and 0.63 ± 0.11 ng/ml, respectively. These results show that full stimulation of TSH by TH deprivation does not require SRC-1.

Sensitivity to TH as determined by the administration of L-T_3

The role of SRC-1 in enhancing the sensitivity of the pituitary thyrotrophs to the suppressive effect of TH is suggested by the higher baseline concentration of serum TSH in SRC-$1^{-/-}$ mice, despite concomitant elevation of serum free T_4 and T_3 levels. To test the degree of hyposensitivity to TH in SRC-$1^{-/-}$ mice directly, these

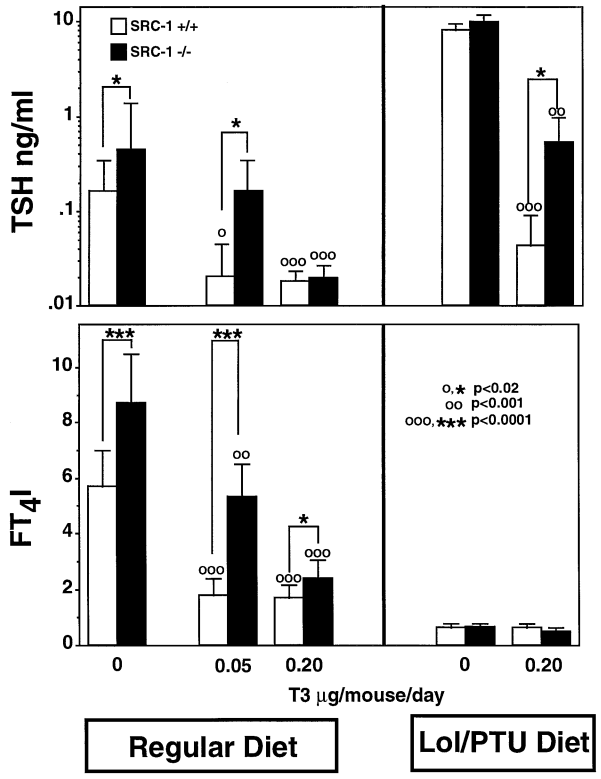

Fig. 2. Effect of L-T_3 treatment on serum TSH (upper panel) and FT_4I (lower panel) concentrations. Data from mice that were fed regular diet are shown on the left panels and those from mice that were given Lo I/PTU diet are on the right panels. There were 10 SRC-$1^{+/+}$ mice and 10 SRC-$1^{-/-}$ in each treatment group fed regular diet and six mice of each type and in each treatment group that received the Lo I/PTU diet. Asterisks identify *p* values for differences between SRC-$1^{+/+}$ and SRC-$1^{-/-}$ mice, and open circles identify *p* values for differences from baseline values before L-T_3 treatment.

mice and SRC-$1^{+/+}$ mice were given two incremental doses of L-T_3 while maintained on a regular diet (Figure 2). The low dose of L-T_3 (0.05 μg/mouse/day) reduced the serum TSH level of SRC-$1^{+/+}$ mice to a mean concentration of 0.02 ± 0.03 ng/ml. Although the same L-T_3 dose given to SRC-$1^{-/-}$ reduced the mean TSH concentration to 0.17 ± 0.16 ng/ml, it remained near the untreated baseline of 0.17 ng/ml in the SRC-$1^{+/+}$ group of mice. In agreement with the TSH data, the low L-T_3 dose had a less suppressive effect on the FT_4I in the SRC-$1^{-/-}$ (5.2 ± 1.4 ng/ml) than SRC-$1^{+/+}$ (1.8 ± 0.6 ng/ml) mice. The higher dose of L-T_3 (0.2 μg LT_3/mouse/day) reduced the serum TSH and FT_4I level to the same degree in both types of mice. These data prove that the increased TH level in SRC-$1^{-/-}$ mice is TSH-dependent and indicate that the radioimmunoassay measures biologically active TSH (Figure 2).

Because the different magnitude of L-T_3-induced TSH suppression between the two types of mice could be attributed, in part, to the higher baseline TH and TSH concentrations in the SRC-$1^{-/-}$ mice, L-T_3 was administered after a sufficient period of TH deprivation to equalize the starting levels of serum FT_4I and TSH. Treatment with 0.2 μg T_3/mouse/day for 4 days resulted in clearly more profound suppression of TSH in SRC-$1^{+/+}$ mice (0.04 ± 0.05 ng/ml) than in SRC-$1^{-/-}$ mice, (0.54 ± 0.43, $p < 0.01$) (Figure 2, right panel).

Discussion

In this study we demonstrate that SRC-1 is an important *in vivo* enhancer of TH-dependent action. Mice deficient in SRC-1 display resistance to thyroid hormone as evidenced by the elevated serum TSH levels despite high serum free T_4 and T_3. The increase in TH concentration is TSH-driven, since suppression of TSH by the administration of supraphysiological doses of L-T_3 resulted in reduction of endogenous T_4 to levels one-tenth of the baseline level. Further evidence for the reduced sensitivity of the thyrotroph to TH was obtained by the demonstration that administration of L-T_3, in doses that almost completely suppress the serum TSH of SRC-$1^{+/+}$ mice, had only a partial suppressive effect on TSH and T_4 in SRC-$1^{-/-}$ mice. A 4-fold higher dose of L-T_3 is required to produce TSH suppression in the SRC-$1^{-/-}$ mouse (0.020 ± 0.10 ng/ml) of equal magnitude as that in the SRC-$1^{+/+}$ mouse (0.016 ± 0.011 ng/ml). Thus as observed previously *in vitro* (Tagami *et al.*, 1997), the co-activator SRC-1 also behaves as a co-repressor *in vivo* when it interacts with a gene that is negatively regulated by TH.

The reduced sensitivity of SRC-$1^{-/-}$ mice to L-T_3 was independent of their higher baseline T_4 and TSH levels because it was also observed when pre-treatment levels of these hormones were equalized in SRC-$1^{-/-}$ and SRC-$1^{+/+}$ mice by induction of hypothyroidism. Notably, the absence of SRC-1 did not affect the full ligand-independent stimulation of TSH. Presumably this effect is mediated through the association of the unliganded receptor with one of the co-repressors (NcoR or SMRT). The latter act as co-activators on genes negatively regulated by TH by a mechanism that is not well understood.

While there is ample *in vitro* evidence that SRC-1 potentiates the effect of TH on the regulation of TR target genes (Jeyakumar *et al*., 1997; Feng *et al*., 1998; Tagami *et al*., 1998), the current study demonstrates for the first time that this effect also holds true *in vivo*. The study also shows that a receptor co-activator other than a TR can produce RTH. This possibility has been suspected previously in one family that fully expressed the RTH phenotype in the absence of mutations in either the TRβ or TRα genes (Weiss *et al*., 1996). Our preliminary data, using crude nuclear extracts from fibroblasts of affected members from this family, suggested the involvement of a protein co-factor that associates with TRβ. It is also likely that the role of SRC-1 in the mediation of the dominant-negative effect of mutant TRβs is more important than currently suspected (Tagami *et al*., 1998).

The precise mechanism whereby SRC-1 deficiency produces RTH remains a matter of speculation. If CBP binds more strongly to the T_3-TR/SRC-1 complex than to the T_3-TR alone, then the absence of SRC-1 should reduce the efficacy of ligand-mediated TR effect. By the same token, in dominantly inherited RTH without TR mutation, the putatively defective SRC-1 molecule could engage the T_3-TR to form an inactive complex that will compete for binding to the target gene promoters and thus exert dominant-negative effect. Furthermore, a state of SCR-1 deficiency may develop in the pituitary gland of subjects with mutant TRβs because of the failure to respond to the TH-mediated upregulation of SRC-1 (Misti *et al*., 1998). Since SRC-1 does not associate with unliganded TRs

(Yoh *et al*., 1997; Collingwood *et al*., 1998; Liu *et al*., 1998; Tagami *et al*., 1998), it is not surprising that serum TSH concentrations were not perturbed in TH-deprived SRC-$1^{-/-}$ mice.

In order to compare the magnitude of RTH in SRC-1 deficient mice with that observed in mice deficient in TRβ (Forrest *et al*., 1996; Weiss *et al*., 1997), TRβ2 isoform only (Abel *et al*., 1998), TRα (Gauthier *et al*., 1999), TRα1 isoform only (Wikstsom *et al*., 1998), and combined TRβ and TRα (Gauthier *et al*., 1999), we graded the relative resistance of the pituitary thyrotrophs to TH. This is expressed as the magnitude of TSH increase, and to lesser extent T_4, relative to those in the corresponding wild-type controls: wild type = TRα1 = TRα < TRβ2 = SRC-1 < TRβ ≪ TRβ and TRα. Activation of TSH in the absence of ligand is not impaired in mice lacking the *SRC-1* or the *TR*β gene (Weiss *et al*., 1997). These results, together with the similarly high TSH levels in the combined TRβ and TRα knock-out mice (Gauthier *et al*., 1999), indicate that neither co-activator nor TRs are required for full expression of TSH.

Materials and methods

Generation and handling of animals

SRC-1 deficient (knock-out or SRC-$1^{-/-}$) mice have a targeted mutation that inserted an in-frame stop codon at the Met381 position and deleted ~9 kb of genomic sequence extending downstream of Met381. This eliminated all SRC-1 functional domains for trasciptional activiation, histone acetyltransferase activity and interactions with nuclear receptors: CBP, P300 and p/CAF (Xu *et al*., 1998). The SRC-$1^{-/-}$ gene defect was maintained on a hybrid genetic background of parental C57B1/6J and 129/SV mouse strains. Heterozygous SRC-$1^{-/+}$ mice were interbred to generate litters containing homozygous SRC-$1^{-/-}$ and SRC-$1^{+/+}$ progeny. The genotype of mice was confirmed by analysis of tail DNA as described previously (Xu *et al*., 1998).

Mice were weaned on the fourth week after birth and were fed Purina Rodent Chow (0.8 p.p.m. iodine) ad libitum and tap water. They were housed, five mice per cage, in an environment with a controlled temperature of 19°C and 12 h alternating darkness and artificial light cycles. All animal experiments were performed according to approved protocols at Baylor College of Medicine and the University of Chicago.

All mice were 60–70 days old at the beginning of each experiment. Weights of SRC-$1^{+/+}$ and SRC$^{-/-}$ mice overlapped and mean ± SD were 25.1 ± 2.7 versus 27.8 ± 1.5 g, respectively. TH deficiency was induced by feeding with low iodine (Lo I) diet supplemented with 0.15% propylthiouracil (PTU) purchased from Harlan Teklad Co. (Madison, WI).

At various intervals, ~300 µl of blood were obtained by retro-orbital vein puncture under light methoxyflurane (Pitman Moore, Mundelein, IL) anesthesia. Experiments were terminated by exsanguination by the same method. Serum was separated by centrifugation and stored at –20°C until analyzed in the same assay for each experiment.

Induction of hypothyroidism and treatment with TH

Thyroid hormone deficiency was induced in male SRC-$1^{-/-}$ and SRC-$1^{+/+}$ mice (12 in each group) by feeding Lo I/PTU diet. On the eleventh day, groups (6 mice each) of SRC-$1^{-/-}$ and SRC-$1^{+/+}$ mice were injected once daily for 4 days with the vehicle only and others received 0.2 µg of L-T_3/mouse/daily (~0.8 µg/100 g body weight/day) while the Lo I/PTU diet was continued. Twelve to 16 h after the last injection, the experiment was terminated by exsanguination. L-T_3, dissolved in phosphate-buffered saline and 0.002% human serum albumin as a vehicle, was given by intraperitoneal injection in a total volume of 0.2–0.3 ml. A stock solution of L-T_3 (Sigma, St Louis, MO) was prepared in water containing 4 mM NaOH and kept at 4°C, protected from light. The concentration of L-T_3 was confirmed by RIA (Diagnostic Products, Los Angeles, CA). Blood samples were obtained at baseline, on the fifth and tenth days after the initiation of the Lo I/PTU diet and at the termination of the experiment on day 14.

In a separate experiment, L-T_3 was given, using the same schedule, to mice with no prior induction of hypothyroidism but in two consecutive incremental doses. The first dose of 0.05 µg L-T_3/mouse/day, given for 4 days, was followed by 0.2 µg L-T_3/mouse/day for 4 additional days. Blood samples were obtained at baseline, before treatment, and 12–16 h after the last dose injection of each incremental L-T_3 dose. There were 10 male mice of each phenotype.

The doses of L-T_3 given to intact and thyroid hormone deficient animals were derived from previous experiments. They were optimized to achieve a partial suppression of serum TSH in order to make evident the differences between wild-type and SRC-$1^{-/-}$ mice. A 4-fold lower dose of L-T_3 was required in intact, as compared to hypothyroid mice to produce a similar suppressive effect on serum TSH.

Measurements of TH and TSH concentrations in serum

Serum TSH was measured in 50 µl of serum using a sensitive, heterologous, disequilibrium double antibody precipitation radioimmunoassay as previously described (Weiss *et al*., 1997). The sensitivity of this assay ranged from 0.01–0.02 ng/ml with intra-assay coefficients of variation of 16, 19 and 10% at 0.04, 0.4 and 4 ng/ml, respectively. Samples containing >5 ng TSH/ml were 5-fold diluted with a TSH-deficient mouse serum.

Serum T_4 and total T_3 concentrations were measured by a double antibody precipitation RIA (Diagnostic Products, Los Angeles, CA) using 25 and 50 µl of serum, respectively. The sensitivity of these assays were 0.2 µg T_4/dl (2.6 nmol/l) and 20 ng T_3/dl (0.5 nmol/l). The inter-assay co-efficients of variation were 5.4, 4.2 and 3.6% at 3.8, 9.4 and 13.7 µg/dl for T_4; and 7.7, 7.1 and 6.2% at 32, 53 and 110 ng/dl for T_3. Free T_4 and free T_3 was estimated by the free indexes (FT_4I and FT_3I) using the respective total hormone values and the resin T_4 uptake test.

Data presentation and statistics

Values are reported as mean ± SD. *p* values were calculated using the Student's *t*-test. Values corresponding to the respective limits of the assays sensitivities were assigned to samples that measured below the detectable range. Only one SRC$^{-/-}$ mouse on Lo I/PTU diet that was given the vehicle died before completion of the experiment. One SRC-$1^{+/+}$ mouse on Lo I/PTU diet that was given L-T_3 had a serum TSH level of 1.63 ng/ml prior to the initiation of treatment and was determined to be an outlier by a two-tailed test with a significance level of <0.05 (Grubbs, 1969; Grubbs and Beck, 1972).

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