

# Decreased collagen mRNA and regression of cardiac fibrosis in the ventricular myocardium of the tight skin mouse following thyroid hormone treatment

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**Objective:** The aim was to study the effect of thyroid hormone on collagen gene expression in the myocardium of the tight skin mouse (TSK), a genetic model of myocardial fibrosis. **Methods:** Heterozygous male (TSK/+) (n=20) and normal (+/+) homozygous mice (n=20), 1.5-2 months old of the C<sub>57</sub> BL/6 strain were studied. Ventricular hypertrophy following thyroid hormone treatment (L-thyroxine, 10 µg·100 g<sup>-1</sup> body weight daily intraperitoneally) was examined by measurement of the heart weight/body weight ratios and histological changes. Expression of fibrillar collagen types I and III in the ventricular myocardium was examined by measurement of the abundance of their respective mRNAs. Collagen synthesis was examined by measurement of hydroxyproline. Deposition of collagen types was evaluated by immunofluorescence staining. Expression of non-collagenous proteins, sarcomeric and cytoskeletal actin, was measured at the mRNA level. **Results:** After 12 days of treatment ventricular hypertrophy was induced in the heart of the TSK mice. The results of northern hybridisation analyses showed that in the hearts of TSK mice 24 h after thyroxine treatment the abundance of mRNA for pro α<sub>2</sub> (I) collagen was decreased by 32% (p<0.05), pro α<sub>1</sub> (III) collagen by 47% (p<0.002), cytoskeletal actin by 50% (p<0.005), and sarcomeric actin mRNA by 34% (p<0.01) compared to the untreated TSK mice. The abundance of mRNA for pro α<sub>2</sub> (I) and pro α<sub>1</sub> (III) collagens in the thyroxine treated TSK mice were nearly comparable to that in normal homozygous mice. In TSK mice which were treated for 12 d, collagen content of the ventricular myocardium, as determined by hydroxyproline measurements, was decreased by 22.5% (p<0.01) compared to that in the heart of normal homozygous mice. **Conclusions:** Effects of thyroid hormone on ventricular gene expression in TSK mice result in a diminished collagen mRNA and collagen content and the disappearance of cardiac fibrosis. Thyroid hormone may selectively prevent the induction of cardiac fibrosis and play an important role in regression of cardiac fibrosis via endocrine pathways.

We have shown previously that expression of collagen types I and III in the heart of the tight skin (TSK) mouse, a genetic mutant with connective tissue abnormalities,<sup>1</sup> is increased at the level of mRNA.<sup>2</sup> By the use of monospecific antibodies to collagen types I and III and immunofluorescent staining, we also showed that collagen type I deposition was increased in perivascular areas of the TSK heart compared to the hearts of normal mice.<sup>2</sup> In those studies we suggested that the heart of the TSK mouse may be used as a genetic model of myocardial fibrosis to study the impact of various pathological and physiological stimuli on the induction or regression of myocardial fibrosis. We have also shown that thyroid hormone treatment of rats leads to the inhibition of collagen gene expression in the ventricular myocardium and in cardiac fibroblasts in culture.<sup>3</sup> The purpose of the present study was to examine the potential regulatory role of thyroid hormone with regard to regression of cardiac fibrosis. We therefore examined the effect of thyroid hormone treatment on gene expression in the ventricular myocardium of the TSK mouse, an established genetic model of myocardial collagen overproduction.

## Animals

Heterozygous male (TSK/+) and normal (+/+) mice, 1.5-2 months old, of the C<sub>57</sub> BL/6 strain from the Jackson Laboratories (Bar Harbor, Maine, USA) were used. TSK/+ and normal mice were maintained on the standard mouse chow diet with access to water and received daily injections (10 µg·100 g<sup>-1</sup> body weight, intraperitoneally) of L-thyroxine. A total of 40 mice were used. All treated mice survived the full course of treatment. For mRNA analyses, treated mice and their age matched control untreated mice, were killed 24 h after treatment. In the case of collagen measurement, TSK mice received daily injections for 12 d. Body weight and ventricular weight were measured at the end of each time point.

## RNA analysis

Total RNA was extracted by the procedure of Chirgwin *et al*<sup>4</sup> with minor modifications as previously described.<sup>2,5</sup> Briefly, hearts were rapidly dissected, atria removed, and ventricular myocardium homogenised in 4M guanidine thiocyanate solution on ice. The homogenate was cleared of cell debris by centrifugation at 10 000 g for 10 min. RNA

was pelleted on a caesium chloride cushion, then redissolved in Tris EDTA, pH 7.4, and purified by three ethanol precipitations. Total RNA was quantified by absorbance at 260 nm assuming  $40 \mu\text{g}\cdot\text{ml}^{-1}$  for each unit of absorbance.

Steady state levels of mRNA were determined by northern hybridisation analysis as previously described.<sup>2</sup> The cDNAs were radioactively labelled by random primer extension as described by Feinberg and Vogelstein,<sup>6</sup> using Amersham Multiprime DNA labelling systems according to the manufacturer's manual (Amersham, England). [ $^{32}\text{P}$ ]-dCTP (specific activity  $300 \text{ Ci}\cdot\text{mM}^{-1}$ , Amersham) was included in the reaction mixture to obtain a specific activity of  $2\text{--}6 \times 10^8 \text{ counts}\cdot\text{min}^{-1}\cdot\mu\text{g DNA}^{-1}$ . Recombinant plasmids used as probes were as follows: rat  $\alpha_2(\text{I})$  sequences specific for the entire 3' non-coding and C-terminal propeptide regions that hybridise to 4.2 and 4.5 kb mRNA<sup>7</sup>; mouse type III collagen DNA fragment that codes for the amino terminal peptide of mouse  $\alpha_1(\text{III})$  collagen<sup>8</sup>; and a full length cDNA for chicken  $\beta$  actin.<sup>9</sup> Prehybridisation, hybridisation, and washing procedures were according to the methods previously described.<sup>2,5</sup> After hybridisation, the membranes were washed and exposed to Kodak XAR-5 film at  $-70^\circ\text{C}$ .

#### Quantitation of hydroxyproline content

Total ventricles (right and left) from treated and control untreated mice hearts were weighed and processed for amino acid analysis as described previously.<sup>10</sup> Briefly, entire ventricular tissue from each heart was hydrolysed in sealed hydrolysis vials with 5.8 N constant boiling HCl for 48 to 72 h at  $106\text{--}108^\circ\text{C}$ . Equal aliquots of each hydrolysate were analysed on a  $\text{W}_2$  ion exchange column and individual peak heights for hydroxyproline were determined for each individual aliquot, in a wavelength of 440 nm. Aminoguanidine aminopropionic acid was used as internal standard. For calculations, the recovery of the internal standard was determined in the aliquots. To convert  $\mu\text{mol}$  of hydroxyproline to mg of collagen, the customary assumption was made that one mole of collagen (molecular weight =  $300\,000$  daltons) contains 300 moles of hydroxyproline; thus  $1 \mu\text{mol}$  of hydroxyproline was equivalent to  $1 \text{ mg}$  of collagen.<sup>10</sup>

#### Immunofluorescent staining

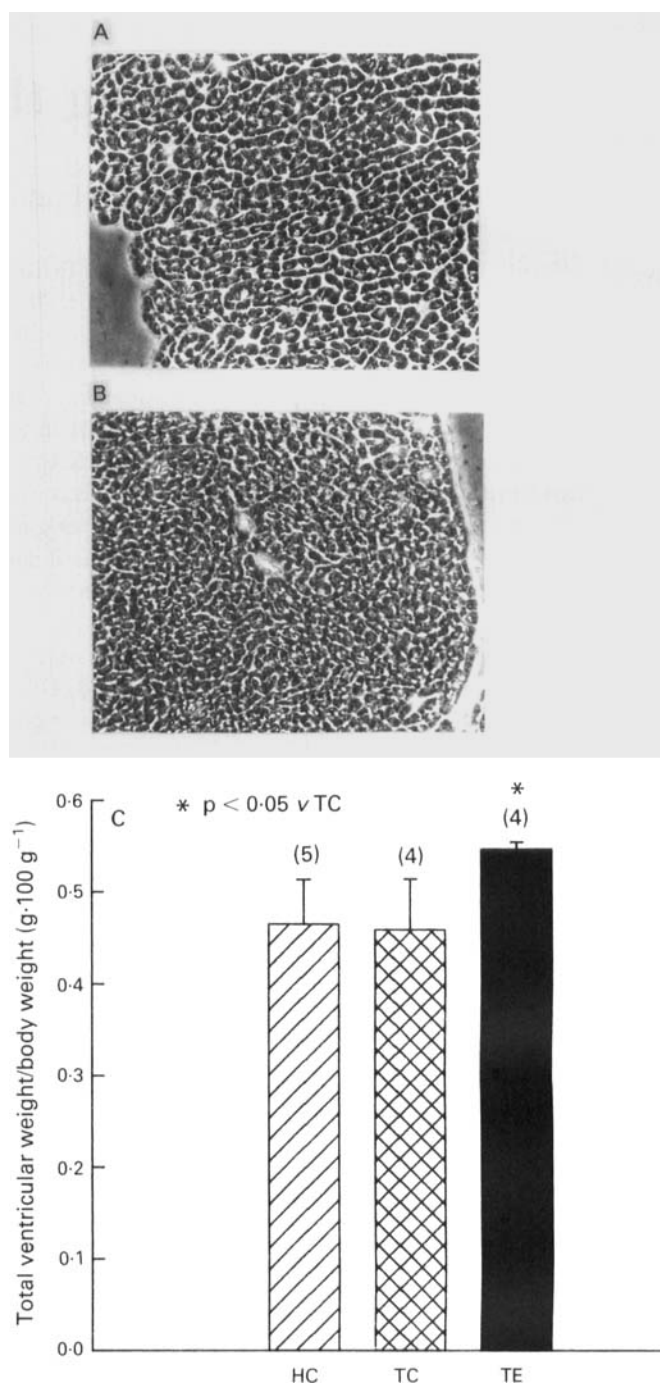
Tissue preparation and staining were performed as previously described.<sup>2,11</sup>

#### Quantification and data analysis

mRNAs were quantified by densitometry scanning (Hoefer) of northern autoradiographs. In addition, ribosomal RNA bands, after transfer to the gene screen, were visualised by ethidium bromide staining and photographed. The density of 28S ribosomal RNA band was measured by densitometry of the negatives. In order to obtain a precise measurement of mRNA per unit total RNA (ie, normalised mRNA), the density of an individual mRNA band was divided by the density of the corresponding 28S ribosomal RNA band. Finally, in order to eliminate inter-gel density differences, all data are presented as a percent of the control value (normalised mRNA for treated group/normalised mRNA for the corresponding control group). It should be noted that samples from a treated group and the corresponding controls were always run on a single gel.

As discussed above, the mRNA measurement, presented as a percent of control, represents the ratio of two means (ie, treated group mean/control group mean). Standard error of this percent mRNA was computed from the standard errors

of measured data using the principle of propagation of error.<sup>12</sup> The percent mRNA data were compared to controls (ie, 100%) by computing the  $t$  statistics, with the significance level adjusted for multiple comparisons using Bonferroni bounds.



**Figure 1** Induction of ventricular hypertrophy in thyroxine treated TSK mice. Heterozygous male (TSK/+) mice (21–25 g) received daily injections ( $10 \mu\text{g}\cdot 100 \text{ g}^{-1}$  body weight intraperitoneally) of L-thyroxine. Mice were killed after 12 days of treatment. Body weight and ventricular weight were measured. Same measurements were made on age matched untreated TSK mice and homozygous (+/+) normal mice. (A) and (B): Phase contrast photomicrographs of frozen sections of control untreated and thyroxine treated mice hearts in cross sectional orientation. Note the enlargement of cardiac myocytes in the thyroxine treated heart section (A). (C): Ventricular weight/body weight in different groups of mice. Columns are means, bars = SD ( $n=4$  or  $5$  in each group). HC = normal homozygous mice; TC = untreated TSK mice; TE = thyroxine treated TSK mice.

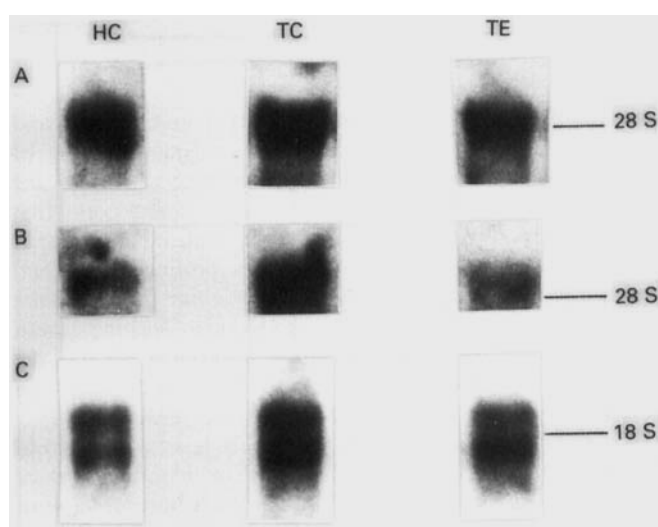
## Results

### Induction of myocardial hypertrophy

We evaluated the induction of cardiac hypertrophy in the thyroxine treated TSK hearts by comparing ratios of the ventricular weight to body weight of treated mice with those of untreated TSK mice. It was shown that thyroxine treatment led to a 19.2% ( $p < 0.05$ ) increase in the heart weight to body weight ratio in the treated TSK mice compared to untreated TSK mice (fig 1). It is noteworthy that the increase in ventricular weight/body weight ratio in thyroxine treated mice was not due to decreased body weight in those animals. Indeed the body weight of the treated mice was increased compared with that of untreated mice. Ventricular hypertrophy in thyroxine treated mice was also evident from the increased dimensions of the cardiac myocytes in histological examination of frozen sections of the heart (fig 1).

### Steady state levels of mRNA

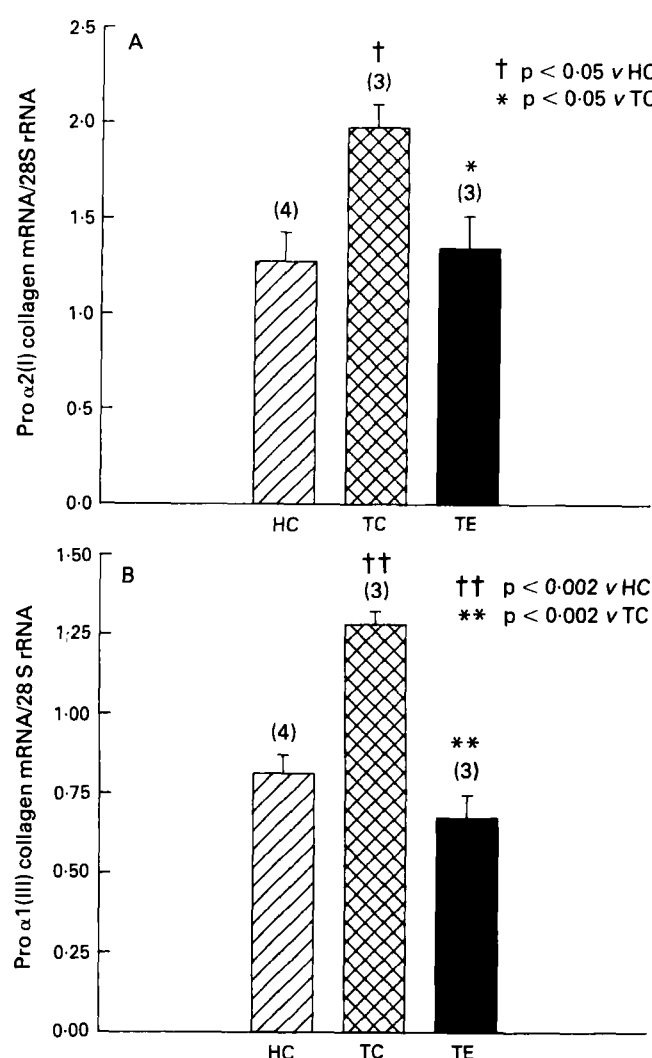
To examine the regulatory effects of thyroid hormone on myocardial collagen gene expression, we measured the abundance of mRNA for pro  $\alpha_2$  (I) and pro  $\alpha_1$  (III) collagens 24 h after treatment with L-thyroxine. This time point was chosen based on our previous results in the rat heart which showed maximum inhibition of collagen mRNA at 24 h after treatment with thyroid hormone. The results of northern hybridisation analysis of RNA from ventricular tissue showed that the abundance of mRNA for pro  $\alpha_2$  (I) collagen in the hearts of the thyroxine treated TSK mice decreased by 32% ( $p < 0.05$ ) compared to the untreated TSK mice. At this time the abundance of mRNA for pro  $\alpha_2$  (I) in treated TSK hearts was indeed comparable to that in normal homozygous mice (figs 2 and 3). It was also shown that thyroid hormone treatment of TSK mice led to decreased (47%,  $p < 0.002$ ) mRNA abundance for pro  $\alpha_1$  (III) collagen in the hearts of treated mice compared to TSK mice which received no treatment (figs 2 and 3). It should be noted that thyroid hormone treatment of homozygous mice also led to decreased abundance of pro  $\alpha_2$  (I) and pro  $\alpha_1$  (III) collagen



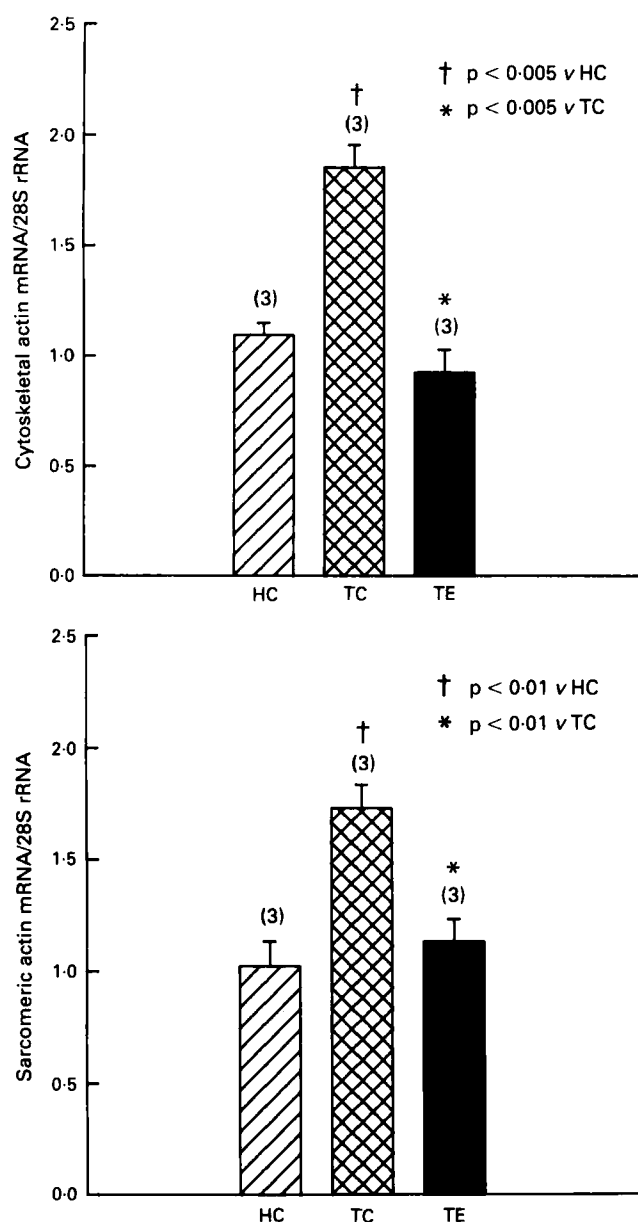
**Figure 2** Northern hybridisation analysis of ventricular RNA for pro  $\alpha_2$  (I), pro  $\alpha_1$  (III) collagen, and actin mRNAs. RNA was extracted from total ventricular myocardium of the thyroxine-treated TSK (TSK/+) and normal (+/+) mice and their respective age matched untreated controls. Electrophoresis, transfer of RNA, hybridisation, and washing procedures were as described in Methods. Autoradiographs A, B, and C are representatives of three individual experiments.

mRNAs in the hearts of these mice compared to untreated homozygous mice.

To understand the impact of thyroid hormone on non-collagenous proteins, we also measured the effects of thyroid hormone treatment on expression of cytoskeletal and sarcomeric actin mRNAs by using a cDNA probe to  $\beta$  actin. This probe, due to high degree of conservation among actin isotypes, hybridises to both sarcomeric and cytoskeletal actin mRNAs. Northern analysis of total ventricular RNA showed that 24 h after treatment with L-thyroxine there was a 50% ( $p < 0.005$ ) decrease in the abundance of mRNA for cytoskeletal actin in the heart of thyroxine treated TSK mice compared to untreated TSK mice (fig 4). The abundance of mRNA for sarcomeric actin was also decreased (34%,  $p < 0.01$ ) in the heart of thyroxine treated TSK mice compared to untreated TSK mice (fig 4).



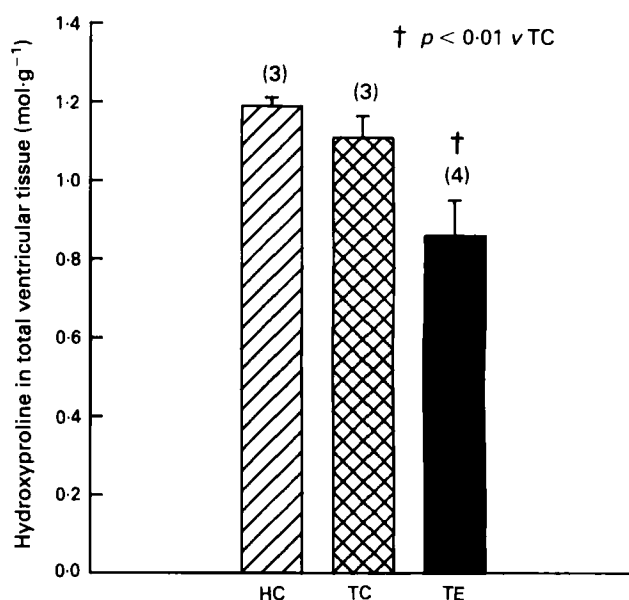
**Figure 3** Results of densitometry scanning of autoradiograms from northern hybridisation analysis of total RNA from ventricular myocardium of mice treated with L-thyroxine and control age matched controls. 10  $\mu$ g RNA from treated and untreated ventricular tissues were applied to 1% agarose gel. Electrophoresis and blotting were performed as described in Methods. Blots were hybridised to  $^{32}$ P labelled cDNA probes to rat pro  $\alpha_2$  (I) (A) and pro  $\alpha_1$  (III) collagen (B). Densitometry scanning was performed to measure the density of mRNA and the corresponding 28S ribosomal RNA bands, as described in Methods. Columns are means, bars=SD of three or four individual experiments. HC=normal control homozygous mice; TC=untreated TSK mice; TE=thyroxine treated TSK mice.



**Figure 4** Results of densitometry scanning of autoradiograms from northern hybridisation analysis of total RNA. 10  $\mu$ g RNA from the ventricular tissue of thyroxine treated (10  $\mu$ g·100  $g^{-1}$  body weight) and untreated TSK (TSK/+) mice and normal homozygous (+/+) untreated mice were electrophoresed on 1% agarose gel. RNA transfer and hybridisation were as described in Methods. Blots were hybridised to  $^{32}P$  labelled full length cDNA probe to chicken  $\beta$  actin. Densitometry scanning was performed to measure the density of mRNAs and their corresponding 28S ribosomal RNA bands as described in Methods. Upper panel shows the results for cytoskeletal actin mRNA. The results for sarcomeric actin mRNA are shown in the lower panel. Columns are means, bars=SD (n=3). HC=normal homozygous mice; TC=untreated TSK mice; TE=thyroxine treated TSK mice.

#### Collagen synthesis

To evaluate whether the decreased abundance of pro  $\alpha_2$  (I) and pro  $\alpha_1$  (III) collagen mRNAs leads to changes in collagen synthesis, we measured the hydroxyproline content of the ventricular myocardium in TSK mice and in their matching control untreated TSK mice following 12 days of treatment with thyroxine. At this time the amount of collagen per gram ventricular tissue, as calculated by hydroxyproline content, was decreased by 22.5% ( $p < 0.01$ ) in the hearts of thyroxine treated TSK mice compared to their age matched



**Figure 5** Effect of thyroxine treatment on collagen content of the ventricular myocardium. Total ventricular tissues from TSK mice treated with L-thyroxine (10  $\mu$ g·100  $g^{-1}$  body weight, intraperitoneally) for 12 d and TSK mice which received no treatment were hydrolysed for 48 h in constantly boiling HCl; hydroxyproline was measured on aliquots of hydrolysate and collagen content was calculated as described in Methods. Columns are means, bars=SD (n=3 or 4). TC=untreated TSK mice; TE=thyroxine treated TSK mice.

controls which received no treatment (fig 5). We also examined the deposition of collagen types I and III by immunofluorescent staining of frozen sections of the TSK mice hearts after 12 days of treatment with thyroxine. Studies of duplicate (in some cases triplicate) hearts showed no considerable differences between the hearts of thyroxine treated TSK mice and those of untreated normal mice, in that focal areas of fibrosis surrounding the blood vessels, usually seen in untreated TSK mice, were absent in thyroxine treated hearts (data not shown).

#### Discussion

The important findings of our study were first, that thyroid hormone treatment of the TSK mice, a genetic model of cardiac fibrosis,<sup>2</sup> led to decreased abundance of mRNA for collagen types I and III in the myocardium; and second, that thyroid hormone treatment resulted in reduced myocardial collagen, as shown by decreased hydroxyproline content and absence of characteristic focal areas of cardiac fibrosis in the myocardium of thyroxine treated TSK mice. Together, these findings may have major biological significance since the heart of the TSK mouse is an established model of ventricular collagen overproduction.<sup>2</sup> Decreased collagen mRNAs and collagen content in such hearts by thyroid hormone has important implications in identification of endocrine pathway(s) that may be involved in regression of myocardial fibrosis. The inhibition of collagen gene expression in the myocardium may be a tissue specific effect of thyroid hormone. Alternatively, this effect may be part of a general effect of thyroid hormone on collagen biosynthesis in various tissues.

Furthermore, the findings of the present study clearly show that ventricular hypertrophy induced by thyroid hormone is not accompanied by cardiac fibrosis. This is in

contrast with the ventricular hypertrophy induced by pressure overload due to abdominal aortic banding<sup>13</sup> or noradrenaline induced high blood pressure.<sup>14</sup> We have shown previously that haemodynamic changes due to pressure overload in the rat heart lead to ventricular hypertrophy with significant myocardial fibrosis.<sup>14</sup> Similarly, noradrenaline infusion led to ventricular hypertrophy, increased collagen gene expression, and myocardial fibrosis.<sup>14</sup> Our studies on collagen gene expression in rabbit cardiac fibroblasts following thyroid hormone treatment have shown that the inhibitory effect of thyroid hormone is mostly at the transcriptional levels.<sup>3</sup> It is also noteworthy that in thyroxine induced hypertrophy ribosomal and messenger RNAs are increased.<sup>15</sup> However, since in the same model, the ratio of total RNA to ribosomal RNA remains unchanged, lack of stimulation or inhibition of gene expression may be the main reason for reduced abundance of collagen mRNA. Decreased collagen content and lack of focal areas of fibrosis in the heart of thyroid hormone treated mice indicate that decreased mRNA levels ultimately lead to decreased collagen deposition in these hearts. It should be noted that decreased collagen content may in part be due to increased collagen turnover.<sup>16</sup> The findings of the present study are therefore of particular importance with regard to identification of different stimuli that could cause various types of cardiac hypertrophy. They also further support our previously stated idea that the distinction should be made between cardiac fibrosis and ventricular hypertrophy and that each phenomenon may be regulated via separate mechanisms.<sup>17</sup> Most importantly, these findings strongly suggest that endocrine pathways in general, and thyroid hormone in particular, may be involved in the regression of cardiac fibrosis.

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**Key terms:** hormone; hypertrophy; heart; extracellular matrix; gene expression

- 1 Green MC, Sweet HO, Bunker LE. Tight-skin, a new mutation of the mouse causing excessive growth of connective tissue and skeleton. *Am J Pathol* 1976;**82**:493-512.
- 2 Chapman D, Eghbali M. Expression of fibrillar types I and III and basement membrane collagen type IV genes in myocardium of tight skin mouse. *Cardiovasc Res* 1990;**24**:578-83.
- 3 Yao J, Eghbali M. Thyroid hormone decreases the steady state levels of mRNA for pro  $\alpha_1$  (I) collagen in the rat myocardium and in cardiac fibroblasts in culture (abstract). *J Cell Biochem* 1991;**15E**:24.
- 4 Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 1979;**18**:5294-9.
- 5 Eghbali M, Czaja MJ, Zeydel M, et al. Collagen chain mRNAs in isolated heart cells from young and adult rats. *J Mol Cell Cardiol* 1988;**20**:267-76.
- 6 Feinberg AP, Vogelstein B. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 1983;**132**:6-13.
- 7 Genovese C, Rowe D, Kream B. Construction of DNA sequences complementary to rat  $\alpha_1$  and  $\alpha_2$  collagen mRNA and their use in studying the regulation of type I collagen synthesis by 1-25-dihydroxy-vitamin D. *Biochemistry* 1984;**23**:6210-6.
- 8 Liao G, Yamada Y, deCrombrughe B. Coordinate regulation of the levels of type III and type I collagen mRNA in most but not all mouse fibroblasts. *J Biol Chem* 1985;**260**:531-4.
- 9 Cleveland DW, Lopata MA, MacDonald RJ, Cowan NJ, Rutter WJ, Kirschner MW. Number and evolutionary conservation of  $\alpha$ - and  $\beta$ -tubulin and cytoplasmic  $\beta$ - and  $\gamma$ -actin genes using specific cloned cDNA probes. *Cell* 1980;**20**:95-105.
- 10 Eghbali ME, Eghbali M, Robinson TF, Seifter S, Blumenfeld OO. Collagen accumulation in heart ventricles as a function of growth and aging. *Cardiovasc Res* 1989;**23**:723-9.
- 11 Eghbali M, Seifter S, Robinson TF, Blumenfeld OO. Enzyme-antibody histochemistry. A method for detection of collagens collectively. *Histochemistry* 1987;**87**:257-62.
- 12 Armitage P. *Statistical methods in medical research*. Oxford: Blackwell, 1971.
- 13 Chapman D, Weber KT, Eghbali M. Regulation of fibrillar collagen types I and III and basement membrane type IV collagen gene expression in pressure overloaded rat myocardium. *Circ Res* 1990;**67**:787-94.
- 14 Bhambi B, Eghbali M. Effect of norepinephrine on myocardial collagen gene expression and response of cardiac fibroblasts after norepinephrine treatment. *Am J Pathol* 1991;**139**:1131-42.
- 15 Zahinger J, Klaubert A. The effect of triiodothyronine on the cardiac mRNA. *J Mol Cell Cardiol* 1982;**14**:559-71.
- 16 Karim MA, Ferguson AG, Wakim BT, Samarel AM. In vivo collagen turnover during development of thyroxine-induced ventricular hypertrophy. *Am J Physiol* 1991;**260**:C316-26.
- 17 Eghbali M, Tomek R, Sukhatme VP, Woods C, Bhambi B. Differential effects of transforming growth factor- $\beta$  on cardiac fibroblasts: regulation of fibrillar collagen mRNAs and expression of early transcription factors. *Circ Res* 1991;**69**:483-90.

