Thyroid Hormone Targets Matrix Gla Protein Gene Associated With Vascular Smooth Muscle Calcification

Yoji Sato, Ryo Nakamura, Mitsutoshi Satoh, Kayoko Fujishita, Satoko Mori, Seiichi Ishida, Teruhide Yamaguchi, Kazuhide Inoue, Taku Nagao, Yasuo Ohno

Abstract—Thyroid hormones have marked cardiovascular effects in vivo. However, their direct effects on vascular smooth muscle cells have been unclear. Because thyroid hormones play critical roles in bone remodeling, we hypothesized that they are also associated with vascular smooth muscle calcification, one of the pathological features of vascular sclerosis. To test this hypothesis, we examined the effects of 3’,5,3,5-triiodo-L-thyronine (T3) on the expression of calcification-associated genes in rat aortic smooth muscle cells (RAOSMCs). Quantitative RT-PCR revealed that a physiological concentration of T3 (15 pmol/L free T3) increased mRNA level of matrix Gla protein (MGP), which acts as a potent inhibitor of vascular calcification in vivo, by 3-fold in RAOSMCs, as well as in cultured human coronary artery smooth muscle cells. In RAOSMCs transiently transfected with a luciferase reporter gene driven by the MGP promoter, T3 significantly stimulated luciferase activity. In addition, RNA interference against thyroid hormone receptor-α gene diminished the effect of T3 on MGP expression. Aortic smooth muscle tissues from methimazole-induced hypothyroid rats (400 mg/L drinking water; 4 weeks) also showed a 68% decrease in the MGP mRNA level, as well as a 33% increase in calcium content compared with that from the control euthyroid animals, whereas hyperthyroidism (0.2 mg T3/kg IP; 10 days) upregulated MGP mRNA by 4.5-fold and reduced calcium content by 11%. Our findings suggest that a physiological concentration of thyroid hormone directly facilitates MGP gene expression in smooth muscle cells via thyroid hormone nuclear receptors, leading to prevention of vascular calcification in vivo. (Circ Res. 2005;97:550-557.)

Key Words: calcium ☐ gene expression ☐ nuclear receptors ☐ vascular smooth muscle ☐ thyroid hormone

Thyroid hormone has marked effects on differentiation, development, and metabolic balance of virtually every body tissue. The action of thyroid hormone is mediated by high-affinity thyroid hormone nuclear receptors (TRs), which recognize specific response elements in the promoters of target genes and regulate their transcriptional activity in response to the hormone. Alterations in thyroid hormone levels have a profound impact on the cardiovascular system, which include changes in myocardial contractility, heart rate, and resistance of peripheral vasculature. Hyperthyroidism leads to positive inotropic, lusitropic, and chronotropic effects on the heart and low systemic vascular resistance, whereas the opposite is observed in hypothyroidism. In myocardium, the mechanisms for these changes are based on altered expression levels of several key proteins involved in the regulation of intracellular ion homeostasis. The effects of thyroid hormone on cardiac contractility as well as rates of contraction and relaxation are mainly mediated by increases in the levels of the sarcoplasmic reticulum Ca2+-ATPase and decreases in its inhibitor phospholamban in cardiomyocytes. The positive chronotropic effect of thyroid hormone is associated with altered expression levels in plasmalemmal ion channels/transporters in the heart, such as Kv1.5, Kv4.2, minK, hyperpolarization-activated cyclic nucleotide-gated channel 2 (HCN2), HCN4, Na+-Ca2+ exchanger, and Na+-K+-ATPase. In contrast, although ÷25% of hypothyroid patients have diastolic hypertension, the mechanism for the altered systemic vascular resistance under an abnormal thyroid hormone status is not well understood. To date, a loss of nongenomic vasodilating action of thyroid hormone and atherosclerosis attributable to hypercholesterolemia have been associated with the increased systemic vascular resistance under hypothyroidism. Recently, mRNAs for TR isoforms were identified in aortic and coronary smooth muscle cells, suggesting that a direct genomic action of thyroid hormone may play a significant role in vascular smooth muscle. Although extremely high concentrations of thyroid hormone are known to regulate expression of several genes invascular smooth muscle cells, the physiological and direct target genes of thyroid hormone in vascular smooth muscle cells are not known.

Arterial calcification is a common pathological feature of vascular sclerosis, as well as a variety of metabolic disorders such as diabetes and renal disease. Decades ago, cretinism
was found to be associated with arterial calcification, especially when patients did not receive sufficient thyroid hormone replacement therapy. However, the mechanism for the calcification in cretins has been to date unclear. A subset of vascular smooth muscle cells, named "calcifying vascular cells," was demonstrated recently to undergo osteogenic and chondrogenic differentiation in culture, indicating that some vascular smooth muscle cells still have the potential for multiple lineages. Because thyroid hormone plays a critical role in bone remodeling, we hypothesized that thyroid hormone is also associated with vascular calcification. To test this hypothesis, we investigated the effect of thyroid hormone on vascular smooth muscle cell calcification and expression profiles of calcification-associated genes in vitro and in vivo, and identified matrix Gla protein (MGP) gene as a target of thyroid hormone in vascular smooth muscle cells.

Materials and Methods

Cell Culture

α-Actin-positive rat aortic smooth muscle cells (RAOSMCs) were obtained from Cell Applications, Inc. and were cultured on 6-well cell culture plates at 37°C in a humidified atmosphere of 95% air/5% CO₂ in growth medium (GM; Dulbecco’s Minimal Essential Medium [DMEM] supplemented with 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin). Cells up to passage 5 were used for the experiments. The culture media were changed every 48 hours. Thyroid hormone–depleted serum was prepared as described previously. To evaluate effects of thyroid hormone on gene expression profiles of the synthetic phenotype of RAOSMCs, the cells at 50% of confluence were cultured in thyroid hormone–depleted medium (TDM; DMEM containing 10% thyroid hormone-depleted serum, 100 U/mL penicillin, and 100 μg/mL streptomycin). Cells up to passage 5 were used for the experiments. The culture media were changed every 48 hours. Thyroid hormone–depleted serum was prepared as described previously. To evaluate effects of thyroid hormone on gene expression profiles of the synthetic phenotype of RAOSMCs, the cells at 50% of confluence were cultured in thyroid hormone–depleted medium (TDM; DMEM containing 10% thyroid hormone-depleted serum, 100 U/mL penicillin, and 100 μg/mL streptomycin) for 2 days and stimulated with 3',5,3'-triiodo-L-thyronine (T₃) for another 2 days.

The contractile form of RAOSMCs was obtained by culturing confluent cells in serum-free differentiation medium (DM; DMEM supplemented with 1 μM ITS-X (Invitrogen), 5 mM L-taurine, 100 U/mL penicillin, and 100 μg/mL streptomycin) for 10 days, followed by T₃ treatment for 2 days. The transition of the cell phenotype in DM was confirmed by immunoblotting for nonmuscle myosin heavy chain (SMemb) and smooth muscle myosin heavy chain-2 (SM2). To examine effects of thyroid hormone on calcium accumulation, confluent RAOSMCs were cultured as described previously in TDM with or without 100 ng/mL recombinant human bone morphogenic protein-2 (rBMP-2; R & D Systems) in a cell culture dish with or without collagen type IV (Col4) coating (BD Biosciences). Supplementation with β-glycerophosphate, which facilitates smooth muscle cell calcification, was omitted because of a decrease in the signal-to-background ratio. Cells were subsequently stimulated with T₃ for 5 days. The concentrations of free T₃ (T₃f) and free L-thyroxine (T₄f) in the serum-containing medium were determined using chemiluminescent enzyme immunoassay at a clinical diagnostic laboratory. The detection limits for T₃f and T₄f were 1.1 pmol/L and 1.7 pmol/L, respectively.

Animals

Male Sprague-Dawley rats (Japan SLC; Shizuoka, Japan) were maintained on rodent chow (Certified diet MF; Oriental Yeast, Co) and given water ad libitum. For generation of hypothyroid animals, methimazole (MMI; 400 mg/L) was added to the drinking water for 4 weeks. Hyperthyroid rats were generated by daily injection of T₃ (0.2 mg/kg body weight IP) for 10 days. Plasma concentrations of T₃ and T₄ were measured, as described above. After the treatment with MMI or T₃, the thoracic aorta was isolated. Animals were 12 weeks old when killed. Aortic smooth muscle tissue for measurements of calcium accumulation and gene expression was cleared of fat, connective tissue, and an endothelium and stored at −80°C until use. For pathological examination, the aortic tissue was fixed in 10% formaldehyde. Transverse aortic sections were taken from the fixed tissue and stained with hematoxylin and eosin. All animals were treated in accordance with laboratory animal care guidelines of National Institute of Health Sciences at Tokyo.

Calcium Accumulation

Calcium content in RAOSMCs and rat aortic smooth muscle tissues were determined as described previously using o- cresolphthalein complexone method. Protein concentration was determined using Bio-Rad protein assay reagent and BSA as a standard.

Real-Time Quantitative RT-PCR

Total RNA was isolated from smooth muscle cells and tissues using Sepasol reagent (Nakalai Tesque) containing 0.1 mg/mL glycogen (Roche Diagnostics) and was treated with DNaseI (Promega) according to the manufacturer protocols. To quantitate specific mRNA levels, the real-time progress of target sequence-specific amplification was monitored during RT-PCR using TaqMan chemistry and PRISM7000 Sequence Detection System (Applied Biosystems). An 18S ribosomal RNA was used as an internal control for each RNA level. Sequences of the primers and the TaqMan probes are listed in supplemental Table I (available online at http://circres.ahajournals.org).

Western Blot Analysis

RAOSMCs and aortic smooth muscle tissues were homogenized in lysis buffer as described previously. After measuring protein concentrations, proteins were separated by SDS-PAGE and blotted onto polyvinylidene fluoride membranes, which were incubated with anti-SMemb or anti-SM2 monoclonal antibodies (Yamasa) or an anti-MGP polyclonal antibody (TransGenic) for 1 hour at room temperature. Subsequently, membranes were incubated with the secondary antibody conjugated with horseradish peroxidase for 1 hour. Signals were visualized and quantified using ECL Plus system (Amersham Biosciences) and LAS-3000 Imaging System (FUJIF-ILM), respectively.

Promoter Activity Assay

Fragments between −1752 and −1 of S' flanking sequence of the MGP gene exon 1 and between −1895 and −1 of S' flanking sequence of the stanniocalcin-1 (STC1) gene exon 1 were amplified using rat tail genomic DNA as a template. The primers for PCR amplifications were designed as based on the nucleotide sequences (MGP forward: CAAGGGTACCGGTTTGAGAGACCACGAGAC; MGP reverse: CTTGGAAGCTCTCTGTGAGTCTGCCTCTGTG; STC1 forward: CAAGGTCGACGGGATATTTCTCGTACGATGCTG; STC1 reverse: CTTGAAAGTTAGGAGACAGTGTATGAGGAG). The amplicons were cloned into the firefly luciferase expression vector pGL3-Basic (Promega). RAOSMCs in the contractile state, grown on a 24-well plate, were transiently cotransfected with 225 ng/well of each promoter luciferase plasmid and 75 ng/well of pGL-TK control plasmid (Promega) using FuGene6 (Roche). Three hours after transfection, cells were incubated with or without T₃. The luciferase activity was defined as a ratio of the firefly luciferase signal to the renilla luciferase signal, which was measured using Dual-Luciferase Reagent (Promega). The transfection efficiency of the plasmids was estimated to be 1% to 10% of the total RAOSMCs, as assessed by transfection experiments with an enhanced green fluorescent protein expression vector pEGFP-N1 (BD Biosciences Clontech; data not shown).

RNA Interference Against TRα

RAOSMCs in the synthetic form were transiently transfected with StealthRNAI (Invitrogen) specific for TRα gene (sense: CCAGAA-GAACCUCUACUCCUCAUU; antisense: AUAGUGGGAUG-
The range of thyroid hormone activity was calculated assuming that \( X\% \times (X/100) \) of T3 is processed to T4 at the site of action in vivo and that the affinity of T4 for TRs is one tenth that of T3. Therefore, we first examined the effects of T3 on mRNA expression of calcification-associated genes in both contractile form of RAOSMCs cultured in DM. Cells were treated with 15 pmol/L or 1 nmol/L T3 for 2 days. Values are expressed as means±SEM, *\( P<0.05 \) vs hypothyroid; #\( P<0.05 \) vs euthyroid.

**Figure 1.** T3 regulated expression of calcification-associated genes in cultured RAOSMCs in synthetic and contractile forms. A, Effect of T3 on mRNA expression of calcification-associated genes in the synthetic form of RAOSMCs cultured in TDM. Cells were treated with 15 pmol/L \( \text{fT}_3 \) for 2 days. B, Effect of T3 on mRNA expression of calcification-associated genes in the contractile form of RAOSMCs cultured in the DM. Cells were treated with 15 pmol/L or 1 nmol/L \( \text{fT}_3 \) for 2 days. Values are expressed as means±SEM (n=4) TH indicates thyroid hormone. *\( P<0.05 \) vs hypothyroid; #\( P<0.05 \) vs euthyroid.
(OPN) was downregulated by 21%. The mRNA levels of BMP2, bone morphogenic protein-4 (BMP4), osteonectin (ON), and core binding factor α1 (Cbfa1; also known as Runx2 or Osf2) were not significantly altered by the T3 treatment. Specific signals for osteocalcin and bone sialoprotein mRNA were not detected by several independent primer sets in all experiments of the present study, presumably because of their low abundance in vascular smooth muscle cells. In the contractile phenotype in DM, T3 (15 pmol/L) induced upregulation of MGP and BMP4 3.2-fold and 1.6-fold, respectively (Figure 1B). A higher concentration of T3 (1 nmol/L) resulted in a further increase in the MGP mRNA level and a significant upregulation of STC1 mRNA, indicating the dose dependency of the effects. Messenger RNA levels of OPN, BMP2, ON, STC1, and Cbfa1 in the contractile phenotype were not significantly altered by the T3 treatment. Among the calcification-associated genes, MGP and STC1 genes were commonly upregulated by T3 in both phenotypes, suggesting that these two genes were relatively essential in RAOSMCs as targets of thyroid hormone.

Because the effect of MGP on calcification and osteogenic differentiation of vascular smooth muscle cells depends on availability of BMP2 and Col4,20,21 cell calcification was determined in the absence or presence of these factors. In the absence of rBMP2 and Col4 coating, treatment with T3 (1 nmol/L total T3=15 pmol/L fT3) for 5 days resulted in an increase in calcium content by 39% in RAOSMCs (Figure 2). The same treatment tended to have the similar effect on cells in a Col4-coated vessel in the absence of rBMP2. In contrast, T3 led to a significant decrease in cellular calcium by 10% in the presence of rBMP2 and Col4-coating.

**Transcriptional Regulation of MGP and STC1 Genes by T3**

To test a hypothesis that the promoters of MGP and STC1 genes were under regulation of thyroid hormone, RAOSMCs were transiently transfected with luciferase reporters under control of the MGP and STC1 promoters. The 5’ flanking sequences of MGP and STC1 genes have been submitted to NCBI (accession numbers AY750958 and AY750959, respectively). Transcription Element Search System (TESS)22 revealed that consensus sequences of the thyroid hormone response element were located in 585 to 600 (TGTACC CCAA TGAACC) and 1009 to 1024 (TGGAGACAGGAGGACA) bases upstream of the putative transcription initiation sites of MGP and STC1 genes, respectively. Treatments of the cells with T3 (15 pmol/L to 1 nmol/L) for 48 hours resulted in significant increases in transcriptional activity compared with vehicle treatment (Figure 3).

**Regulation of MGP and STC1 Expression via TRs**

Arterial smooth muscle cells express TRα1 and TRα2 nuclear receptor isoforms strongly and TRβ1 and TRβ2 relatively weakly.10 To evaluate the involvement of TRs in the T3-induced upregulation of MGP and STC1 mRNAs, RNA interference (RNAi) was performed using StealthRNAi specific for the TRα gene encoding TRα1 and TRα2. The RNAi in RAOSMCs for 2 days significantly attenuated mRNA expression of TRα1 and TRα2 by 66% and 57%, respectively, compared with the controls (Figure 4). The RNAi was also associated with upregulation of MGP mRNA by 32%.
Hypermethroidism elicits pronounced vascular relaxation. However, the effect of hyperthroidism on vascular calcification has been unclear. Therefore, it is of interest to compare effects of hyperthyroidism on the expression profiles of calcification-associated genes with those of hypothyroidism. In contrast to the aortic smooth muscle from hypothyroid rats, daily injections of T3 for 10 days led to a decrease in the calcium content in the rat aortic smooth muscle tissues by 11% compared with that of euthyroid animals (Figure 6A). Quantitative RT-PCRs showed that mRNA levels of MGP, OPN, and BMP2 were upregulated by 4.5-fold, 4.9-fold, and 3.4-fold, respectively, by the T3 treatment, whereas hyperthyroidism resulted in a significant decrease in the level of STC1 mRNA (Figure 6B).

Upregulation of MGP mRNA in Cultured HCASMCs

To demonstrate that thyroid hormone regulates MGP gene expression in vascular smooth muscle cells of a different species, we determined MGP mRNA levels in HCASMCs in the presence and absence of T3. Treatment with a physiological concentration (15 pmol/L) of T3 for 2 days led to a significant increase in MGP mRNA by 40% (1.0 ± 0.07 [hypothyroid 0 pmol/L T3] versus 1.40 ± 0.13 [euthyroid 15 pmol/L T3]) in an arbitrary unit; n = 12).

Discussion

In the present study, thyroid hormone led to an upregulation of MGP in arterial smooth muscle cells in vitro regardless of culture condition, phenotype, and animal species of the cells. The transcriptional activity of the MGP gene was increased by T3, and reduction of TRα gene expression led to a loss of responsiveness of the MGP gene to T3, suggesting that the effect of T3 is based on a genomic action via TRα1. Furthermore, in vivo hormone levels were positively and negatively associated with the expression of MGP and calcification in vascular smooth muscle, respectively. Because aortic smooth muscle from hypothyroid rats showed no obvious neointimal formation, the vascular calcification under the hyperthyroidism is likely to be similar to that of media sclerosis. In aortic smooth muscle from hypothyroid rats, expression levels of calcification activators BMP4, ON, and Cbfa1 were decreased, whereas hyperthyroidism upregulated another calcification activator BMP2. However, calcium content in aortic smooth muscle was increased in hyperthyroidism, suggesting that the expression or function of calcification inhibitors, such as MGP and OPN, are more dominant for the phenotypic outcome in vivo, compared with those of the calcification activators.

MGP is a mineral-binding extracellular matrix protein synthesized by vascular smooth muscle cells and chondrocytes. Luo et al have shown that ablation of MGP gene in mice causes extensive and lethal calcification and cartilaginous metaplasia of the media of all elastic arteries, indicating that MGP has an inhibitory effect on media calcification in vivo. In contrast, in the same study, morphological analysis showed that heterozygous MGP knockout mice, which had a

Calcium Accumulation in Hypothyroid Rat Aorta

As examined by hematoxylin-eosin staining, the cross-sections of aorta from rats treated with MMI for 4 weeks did not show obvious calcified foci (Figure 5A). However, α-cresolphthalein complexone experiments indicated that the 4-week treatment with MMI significantly increased the calcium content in the rat aortic smooth muscle tissues by 33% compared with that of euthyroid animals (Figure 5B). Quantitative RT-PCRs revealed that mRNA levels of MGP, BMP4, ON, and Cbfa1 were downregulated by 68%, 87%, 69%, and 72%, respectively, by the MMI treatment. OPN, BMP2, and STC1 mRNA levels were not significantly altered (Figure 5C). MMI also attenuated protein expression of MGP by 54% (Figure 5D). Calcified foci were not observed even in aortic cross-sections from rats treated with MMI for 12 weeks (online Figure II), suggesting that the calcification was not progressive.

Calcium Content in Hyperthyroid Rat Aorta

The RNAi was associated with loss of responsiveness of MGP (Student t test [A] or Student-Newman–Keuls test [B]); #P < 0.05 (Student t test [A] or Student-Newman–Keuls test [B]); #P < 0.05 association with RNAi (two-way ANOVA).
similar decrease in an arterial MGP level to that by the MMI treatment in the present study, had no obvious calcified foci in arterial smooth muscle. However, this does not necessarily imply that the heterozygous ablation of MGP gene does not affect calcium content in arteries because biochemical quantification for tissue calcium has not been performed. Thus, it is still possible that there is a dose-response relationship between MGP and media calcification, and that an \( \approx 50\% \) reduction of MGP results in some increase in calcium content, although it may not be morphologically evident.

The extracellular environment around smooth muscle cells is known to determine a functional role of MGP. Namely, calcification of vascular smooth muscle cells in the presence of a relatively high concentration of BMP2 was inhibited by MGP, whereas calcification of vascular smooth muscle cells under a low concentration of BMP2 was stimulated by MGP.\(^{20}\) Moreover, extracellular matrix proteins, especially Col4, have significant influence on MGP function and vascular calcification.\(^{21}\) In fact, the effect of T3 on calcification of RAOSMCs was determined by these environmental factors. Therefore, these results suggest that T3 regulates smooth muscle cell calcification, at least partly, by promoting MGP expression, although not only vascular cells but also migratory adventitial pericytic myofibroblasts and circulating skeletal progenitors may have some additional contributions to vascular calcification in vivo.\(^{24}\)

STC1 is a mammalian homolog of stanniocalcin, the fish calcium/phosphate-regulating polypeptide that inhibits calcium flux into cells and stimulates phosphate reabsorption. In the present study, gene transcription of STC1 appeared to be regulated by T3 via TR\( \alpha \) in a dose-dependent manner. However, the highest mRNA expression of STC1 tended to be achieved at a euthyroid status in vitro and in vivo, and hyperthyroidism significantly attenuated the STC1 mRNA expression in vivo. Recently, STC1 was shown to accelerate osteoblast development in an autocrine/paracrine manner in cultured fetal rat calvaria cells.\(^{25}\) Therefore, the downregulation of STC1 may also contribute to the low calcium content in aortic smooth muscle of hyperthyroid rats, although the mechanism that offsets the increase in the STC1 gene transcription remains to be elucidated.

OPN is known to inhibit or promote vascular smooth muscle calcification in vivo in a phosphorylation-dependent manner.\(^{26}\) Therefore, the changes in its expression in the synthetic form of RAOSMCs and in smooth muscle tissue of hyperthyroid rats may be also associated with the effect of thyroid hormone on calcium accumulation. However, as shown in the in vitro and MMI experiments, a physiological concentration of thyroid hormone is unlikely to target OPN gene directly, at least in the contractile form of aortic smooth muscle cells.

BMP2 was upregulated by hyperthyroidism in vivo, whereas BMP4 was downregulated by MMI-induced hypothyroidism. BMP2 is known to antagonize the effect of MGP,\(^{20}\) and BMP4 has been suggested to play a significant role as a cytokine, a growth factor or a media-calcification promoter in vascular lesions of calciphylaxis.\(^{27}\) The mRNA levels of ON and Cbfa1 were also decreased in hypothyroid rat aortic smooth muscle. With the exception of BMP4, these changes in vivo did not follow on from the in vitro experiments,
Increased vascular stiffness underlies the high systemic vas- 
ular resistance observed in hypothyroidism. Vascular calcification 
can lead to some other serious problems, including 
vacular stenosis, calciphylaxis, and even sudden death. Recently, a polymorphism in the promoter region of MGP 
gene was found to have a significant association with myo- 
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Addition, a recent meta-analysis of coronary artery calcium 
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further studies on the protective role of thyroid hormone and 
MGP against vascular smooth muscle calcification should 
provide insights into novel therapeutic strategies for the high 
systemic vascular resistance and blood pressure of hypothy- 
roid patients, as well as for diseases associated with cardio- 
vascular calcification such as diabetes, chronic renal insuffi- 
ciency, and hypercholesterolemia.

Figure 6. Hyperthyroidism decreased tissue calcium content in 
aortic smooth muscle and altered the expression profile of 
calcification-associated genes. Hyperthyroidism was achieved 
by daily injection of T3 (0.2 mg/kg body weight IP) for 10 days. 
A. Effect of the hyperthyroidism on calcium deposition in rat 
aortic smooth muscle. C. Effect of hyperthyroidism on mRNA 
expression of calcification-associated genes in rat aortic smooth 
muscle. Values are expressed as mean±SEM (n=7 to 8); 
*P<0.05 vs euthyroid.

suggested that the alterations were not attributable to a direct 
effect of thyroid hormone on vascular smooth muscle cells. 
Because physiological concentrations of thyroid hormones 
upregulated BMP4 in the contractile form of RAOSMCs and 
and in vivo (hypothyroid versus euthyroid), BMP4 may be 
another direct target of thyroid hormone in aortic smooth 
muscle cells. However, the expected influences of the 
changes in all the calcification activators above were appar- 
etly masked, aforementioned, indicating their minor roles in 
vascular calcification, compared with those of calcification 
inhibitors.

In summary, our findings, for the first time, demonstrate 
that a physiological concentration of thyroid hormone has 
significant genomic effects on vascular smooth muscle cells 
in vitro and in vivo, which are associated with vascular 
calcification. Most notably, a decrease in thyroid hormone 
and the concomitant increase in vascular calcification in vivo 
are marked by a decrease in the level of MGP expression, 
suggesting that a physiological concentration of thyroid 
hormone has a direct protective role against vascular smooth 
muscle calcification in vivo. Although vascular calcification 
has been thought to be benign, arterial calcification should 
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References

1. Carr AN, Kranias EG. Thyroid hormone regulation of calcium cycling 
2. Abe A, Yamamoto T, Isome M, Ma M, Yaoita E, Kawasaki K, Kihara I, 
Aizawa Y. Thyroid hormone regulates expression of shaker-related 
potassium channel mRNA in rat heart. Biochem Biophys Res Commun. 
Giles W, Chassande O, Samarut J, Dillmann W. Cardiac ion channel 
expression and contractile function in mice with deletion of thyroid 
4. Boerth SR, Artman M. Thyroid hormone regulates Na–Ca2+ exchanger 
expression during postnatal maturation and in adult rabbit ventricular 
5. Orlowski J, Jingrel JB. Thyroid and glucocorticoid hormones regulate 
the expression of multiple Na,K-ATPase genes in cultured neonatal rat 
6. Klein I, Ojamaa K. Thyroid hormone and the cardiovascular system. 
7. Ojamaa K, Klemperer JD, Klein I. Acute effects of thyroid hormone on 
Subclinical hypothyroidism is an independent risk factor for atheroscle- 
orosis and myocardial infarction in elderly women: the Rotterdam Study. 
9. Klein I, Ojamaa K. Thyroid hormone: targeting the vascular smooth 
10. Mizumura H, Murakami M, Mori M. Thyroid hormone activation in human 
vascular smooth muscle cells: expression of type II iodothyronine deio- 
11. Fukushima K, Ichiki T, Takeda K, Tokunou T, Iino N, Masuda S, 
Ishibashi M, Egashira K, Shimokawa H, Hirano K, Kanaide H, Takeshita 
A. Downregulation of vascular angiotensin II type 1 receptor by thyroid 
L. Multilineage potential of cells from the artery wall. Circulation. 


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Representative images of immunoblots for SM2 (smooth muscle myosin heavy chain-2) and SMemb (nonmuscle myosin heavy chain) in RAOSMCs before and after treatment with the differentiation medium for 10 days. The differentiation medium altered the protein expression of SM2 and SMemb by $11.6 \pm 2.8$ fold ($n=5$) and $0.28 \pm 0.04$ fold ($n=4$), respectively.
Hematoxylin-Eosin staining of cross sections of aortas from a hypothyroid rat treated with methimazole (MMI, 400 mg/L drinking water) for 12 weeks and a euthyroid control rat. Bar=30µm.
<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>TaqMan Probe</th>
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<tbody>
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<td><strong>BMP2</strong></td>
<td>5’-TGAACACAGCTGGTCTCAGGTAA-3’</td>
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<td>5’-AGTGACTTTTGGCCACGACGTTAAAGG-3’</td>
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<td><strong>BMP4</strong></td>
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<td><strong>MGP (rat)</strong></td>
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<tr>
<td><strong>MGP (human)</strong></td>
<td>5’-TTCATATCCCTCTCAGGAGATG-3’</td>
<td>5’-TCGATTATGAGCAGATTGTATCC-3’</td>
<td>5’-AACGCTCTAAGCCTGTCCACAGGCTCAATA-3’</td>
</tr>
<tr>
<td><strong>ON</strong></td>
<td>5’-CACCTGAAGCTCATCAGGACAT-3’</td>
<td>5’-TTGTTGCCTCATCTCTCTGTA-3’</td>
<td>5’-TGATGCTGGCTAAAACAGCTTGTGT-3’</td>
</tr>
<tr>
<td><strong>OPN</strong></td>
<td>5’-TGACCATCAGGAGCTGATG-3’</td>
<td>5’-GCTTGTGCCCTCATCTCTCTGTA-3’</td>
<td>5’-ACGGAGACCATGAGAGAGGAGT-3’</td>
</tr>
<tr>
<td><strong>STC1</strong></td>
<td>5’-GGATCACCTCCAAGGTCTTCCT-3’</td>
<td>5’-GGCAATGCTGCAAACATTGA-3’</td>
<td>5’-TTCGGAGGTGTTCTACTTTCCAGAGGATG-3’</td>
</tr>
<tr>
<td><strong>TRα1</strong></td>
<td>5’-AGCTGCTGATGAAAGGTAAGTG-3’</td>
<td>5’-GCTTAGTCTTGCTGGCGAGTA-3’</td>
<td>5’-ACGGAGACCATGAGAGAGGAGT-3’</td>
</tr>
<tr>
<td><strong>TRα2</strong></td>
<td>5’-GGCAATACCTTGTCCCTTTGAG-3’</td>
<td>5’-CCACGTAAGCAGCAACTATTTC-3’</td>
<td>5’-ACTCAAGTGTCACCTCCTCCAGCTC-3’</td>
</tr>
</tbody>
</table>