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Thyroid hormone non-genomically suppresses Src thereby stimulating osteocalcin expression in primary mouse calvarial osteoblasts

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ABSTRACT

To provide further insights into non-genomic action of thyroid hormone (T3), we investigated whether Src is under control of T3 in primary calvarial osteoblasts prepared from neonatal mice. Treatment of the cells with T3 rapidly decreased Src Y416 autophosphorylation, followed by the decrease of phosphorylated extracellular signal-regulated kinases, suggesting that T3 non-genomically suppresses Src activity. Furthermore, this T3 effect was rapid and persistent, and was associated with the increased expression of osteocalcin (OC). To confirm the contribution of Src to the effect of T3 on OC expression, a constitutively active Src (Y527F) was overexpressed in osteoblasts. In such cells, Y416 phosphorylation was markedly increased even in the presence of T3, and T3-dependent expression of OC was markedly attenuated. The present study demonstrates a novel, non-genomic action of T3 in primary mouse osteoblasts, by which T3 suppresses Src thereby stimulating OC expression.

The active thyroid hormone, 3,3′,5-triiodo-L-thyronine (T3), binds to the nuclear thyroid hormone receptor (TR), which functions as a ligand-dependent transcription factor and controls expression of the target genes. In mammals, there are several TR isoforms, such as TRα1, TRβ1, and TRβ2. A number of T3-responsive genes have been identified. However, increasing evidence indicates that not all of the biological effects of T3 are mediated by the genomic action [1]. Some effects can be attributed to the non-genomic action, which includes the regulation of kinase cascades [2,3]. Recently we reported a novel, non-genomic action of T3, through which the pathway consisting of phosphoinositide 3-kinase (PI3K)—Akt—mammalian target of rapamycin (mTOR) was activated in primary human skin fibroblasts [4].

Src is a non-receptor tyrosine kinase, which is expressed in a wide variety of tissues [5]. Src is controlled by receptor protein–tyrosine kinases, G-protein-coupled receptors, cytokine receptors, and nuclear steroid hormone receptors such as estrogen receptor. The regulation of Src kinase activity by phosphorylation has been well characterized [6]. One of the important phosphorylation sites is Y527 that is located near the C-terminus. Under basal conditions, Y527 is phosphorylated, and the phospho-Y527 interacts with Src SH2 domain intramolecularly, rendering the enzyme a dormant form. The Y527F mutant, that cannot be phosphorylated at the 527 site, is more active than the wildtype enzyme, and can induce tumors *in vivo*. Another well characterized phosphorylation site is Y416 that is present within a kinase domain. The autophosphorylation of Y416 augments kinase activity.

Src kinase signaling contributes to the regulation of bone development and remodeling [7,8]. Recently, it is shown that Src plays an important role in differentiation of not only osteoclasts but also osteoblasts [9]. Decreased expression of Src enhances the differentiation of osteoblasts. On the other hand, T3 is also well known to regulate bone remodeling [10,11]. T3 stimulates the expression of several differentiation markers in osteoblasts, including osteocalcin (OC) and alkaline phosphatase (ALP) [12,13]. In this study, to provide further insights into non-genomic action of T3, we investigated whether Src is under control of T3, by utilizing primary calvarial osteoblasts prepared from neonatal mice.

Materials and methods

Primary culture of mouse calvarial osteoblasts. The experimental protocol was approved by Committee for Animal Experiment of Research Institute of Environmental Medicine, Nagoya University. Mouse calvarial osteoblasts were prepared from calvaria of neonatal mice [14]. Calvaria were removed from the animals under aseptic conditions and incubated at 37 °C in Dulbecco's modified Eagle medium (DMEM) containing trypsin (0.5 mg/mL, Gibco, Grand Island, NY) and ethylene-diamine tetraacetic acid (EDTA; 1.5 mg/mL) under continuous agitation. Trypsin digests were discarded at 15 min and replaced with DMEM containing 1 mg/mL of collagenase (Wako, Osaka, Japan). The collagenase digests were discarded at 20 min and replaced with fresh enzyme dilution. The cells released at

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between 20 and 40 min were collected and cultured in proliferation medium [DMEM supplemented with 20% fetal bovine serum (FBS; Hyclon Laboratories, Logan, UT)]. To detect the effects of T3, the cells were cultured until 80% confluence and were incubated for 12 h in DMEM supplemented with 1% charcoal/dextran-treated FBS, which was depleted of hormones and growth factors, before treatment with 10 nM T3. A selective inhibitor of Src family tyrosine kinases, 4-amino-5-(4-methylphenyl)-7-(tbutyl)pyrazolo [3,4-d] pyrimidine (PP1; 2 μM; Biomol, Plymouth Meeting, PA), and protein–tyrosine phosphatase inhibitors, such as sodium molybdate (SM; 2 μM; Sigma–Aldrich, St. Louis, MO), phenylarsine oxide (PAO; 20 nM; Sigma), and protein–tyrosine phosphatase inhibitor III (PTPI-III; 10 μM; Calbiochem, San Diego, CA), were added 1 h before T3 stimulation.

Western blot analysis. The procedures for preparation of whole cell lysates and Western blot analysis were described in our previous report [4]. In brief, whole cell lysates (30 μg/lane) were separated by SDS–PAGE, and transferred onto polyvinylidene difluoride membrane (Amersham Pharmacia, Piscataway, NJ). The blots were probed with the first antibodies described below, followed by incubation with horseradish peroxidase-conjugated, anti-rabbit IgG antibody. Rabbit anti-phospho-Src (Y416), anti-phospho-Src (Y527), anti-Src, and anti-phospho-extracellular signal-regulated kinases (Erk1/2, p44/42 MAPK) (T202/Y204) antibodies were purchased from Cell Signaling (Beverly, MA). Anti-Runt-related transcription factor (RUNX) 2 and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The proteins were visualized using enhanced chemiluminescence (ECL) reagents (Pierce, Rockford, IL). Images of the membranes were obtained using an LAS-1000 lumino-image analyzer (Fuji film, Tokyo, Japan), and densitometric analysis was performed using a software on the LAS-1000 system.

RT-PCR. Total RNA was extracted using a kit (ChargeSwitch Total RNA Cell kits, Invitrogen, Carlsbad, CA). RT-PCR was performed using following primers: 5′-AAGCAGGAGGGCAATAAGGT-3′ and 5′-AGCTGCTGTGACATCCATAC-3′ for OC; 5′-CCGCACGACAACCGCAC CAT-3′ and 5′-CGCTCCGGCCCACAAATCTC-3′ for RUNX2; 5′-GCCC TCTCCAAGACATATA-3′ and 5′-CCATGATCACGTCGATATCC-3′ for ALP; 5′-GGACCTGATACATGTTGCTA-3′ and 5′-GAC TTTCATGTGGAG GAAGC-3′ for TRα1; 5′-CAAGAAGGTGGTGAAGCAGG-3′ and 5′-TCC ACCACCCTGTTGCTGTA-3′ for GAPDH.

Measurement of alkaline phosphatase activities. The cells were seeded into 96-well plates at a density of 1.5×10^4 cells per well, and cultured for 24 h. The cells were then treated with 10 nM T3 and/or PP1 for 5 days in DMEM supplemented with 1% charcoal/dextran-treated FBS. At the end of the incubation, ALP activity in the cells was measured by a *p*-nitro phenyl-phosphate method using a commercially available kit (LABOASSAY™ ALP, Wako).

Transfection. The cells were cultured in 35-mm dishes until 80% confluence, and were transfected with the plasmids expressing constitutively active Src (Y527F) [15] and wildtype Src (WT), using Lipofectamine™ Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, PLUS™ Reagent (Invitrogen) was mixed with DMEM containing 2 μg of plasmid DNA, and incubated for 15 min at room temperature. The diluted Lipofectamine Reagent was then added to the plasmid/reagent mixture, and further incubated for 15 min. The complex containing 2 μg of plasmid was added to the cells, and incubated for 5 h at 37 °C. The transfection medium was then replaced with fresh proliferation medium. After 24-h incubation, the transfected cells were incubated for 12 h in DMEM supplemented with 1% charcoal/dextran-treated FBS before T3 treatment, or were treated with T3 in DMEM supplemented with 1% charcoal/dextran-treated FBS for 36 h.

Statistical analysis. Statistical analysis was performed by Student's *t*-test, and *p* value less than 0.05 was considered to be significant.

Results

T3 rapidly suppresses Src in primary mouse calvarial osteoblasts

Mouse calvarial osteoblasts prepared from neonatal mice were treated with 10 nM T3, and whole cell lysates were subjected to Western blot analysis to determine the levels of Y416 and Y527 phosphorylation in Src. As shown in Fig. 1A, the levels of Y416 autophosphorylation were decreased as early as 5 min in response to T3. By contrast, the levels of Y527 phosphorylation were not altered by T3. The rapid effect of T3 suggested that T3 non-genomically suppresses Src kinase activity. Indeed, the levels of T202/Y204 phosphorylation in Erk1/2, which is downstream of Src, were decreased within 30 min, following the decrease of Y416 autophosphorylation. In addition, treatment with 1–100 nM T3 for 10 min decreased the levels of Y416 autophosphorylation, significantly and dose-dependently.

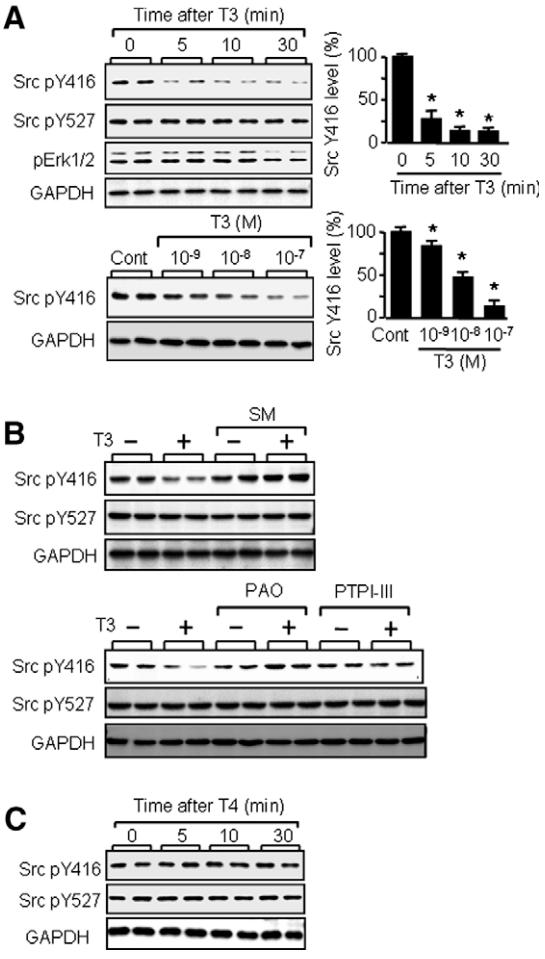

Fig. 1. T3 rapidly suppresses Src in mouse calvarial osteoblasts. (A) Serum-starved, mouse calvarial osteoblasts were treated with 10 nM T3 for indicated times, or osteoblasts were exposed to 1–100 nM T3 for 10 min. Whole cell lysates were subjected to Western blot analysis using antibodies against phospho-Src (Y416), phospho-Src (Y527), phospho-Erk1/2 (T202/Y204), and GAPDH. Experiments were performed in duplicate culture. In densitometric analysis, the phospho-Src levels were normalized by the GAPDH levels, and expressed as percentage of the levels in the cells without T3 (time 0 or Control). Mean ± SD ($n = 4$). *$p < 0.05$ vs. the control levels. (B) Osteoblasts were treated for 10 min with 10 nM T3 in the presence and absence of sodium molybdate (SM, 2 μM), phenylarsine oxide (PAO, 20 nM), or protein–tyrosine phosphatase inhibitor III (PTPI-III, 10 μM). They were added 1 h before T3 stimulation. Whole cell lysates were subjected to Western blot analysis. (C) Osteoblasts were treated with 10 nM T4 for indicated times, and Western blot analysis was performed.

We next studied the possible effects of inhibitors of protein–tyrosine phosphatase (PTP), such as SM, PAO, and PTPI-III. As shown in Fig. 1B, treatment of primary osteoblasts with these inhibitors prevented the T3-dependent suppression of Y416 autophosphorylation, suggesting that some phosphatase(s) may be involved in the suppressive effect of T3 on Src.

On the other hand, it has been reported that T4 as well as T3 exerts a non-genomic effect in some models [16,17]. However, in our model, T4 has no effects on the levels of Y416 and Y527 phosphorylation (Fig. 1C).

Long-term effects of T3 on calvarial osteoblasts

To study whether suppression of Src leads to differentiation of osteoblasts, we first examined the long-term effects of T3 on the levels of OC, ALP, RUNX2, and TRα1 mRNAs by RT-PCR. RUNX2 is a pivotal transcription factor for the OC gene [18]. As shown in Fig. 2A, treatment of the cells with 10 nM T3 for 36 h resulted in the increased levels of OC and ALP mRNAs. By contrast, the levels of RUNX2, TRα1, and GAPDH were not changed. Among TR isoforms, TRα1 was found to be the main isoforms in calvarial osteoblasts (data not shown). In addition, at the protein levels, RUNX2 was not affected by T3 (Fig. 2B). Notably, a marked decrease in the phosphorylation of Src Y416 was observed, indicating that the suppressive effect of T3 on Src is rapid and persistent.

Effects of PP1, SM, PAO, and PTPI-III on T3-dependent expression of OC

We next studied the effect of a selective inhibitor of Src, PP1, on the expression of OC. As shown in Fig. 3A, treatment with PP1 significantly increased OC mRNA, the level of which is similar to that by T3. However, no additional increase was observed by the combined treatment with PP1 and T3. The effects of PP1 on ALP activities were also studied (Fig. 3B). T3 significantly increased ALP activities, and PP1 also stimulated them. However, no additional effect was observed in the combined treatment. These results indicate that OC expression and ALP activities in mouse calvarial osteoblasts are at least partially under control of Src.

We also studied the effects of PTP inhibitors, SM, PAO and PTPI-III. As shown in Fig. 3C, these inhibitors attenuated the T3-dependent increase of OC mRNA.

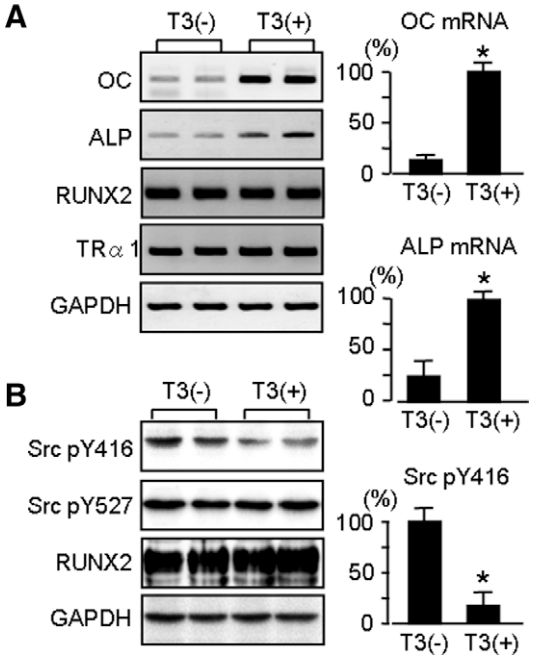

Fig. 2. Long-term effects of T3 on osteoblasts. (A) Mouse calvarial osteoblasts were treated with 10 nM T3 for 36 h, and total RNA was subjected to RT-PCR to analyze the expression of OC, RUNX2, ALP, TRα1, and GAPDH. Experiments were performed in duplicate culture. In densitometric analysis, OC and ALP mRNA levels were normalized by the GAPDH levels, and expressed as percentage of the levels in the cells treated with T3. Mean ± SD ($n = 4$). $^*p < 0.05$ vs. the levels in the cells without T3. (B) Osteoblasts were treated as described above, and whole cell lysates were subjected to Western blot analysis using antibodies against phospho-Src (Y416), phospho-Src (Y527), RUNX2, and GAPDH. Experiments were performed in duplicate culture. In densitometric analysis, the phospho-Src (Y416) levels were normalized by the GAPDH levels, and expressed as percentage of the levels in the cells without T3. Mean ± SD ($n = 4$). $^*p < 0.05$ vs. the levels in the cells without T3.

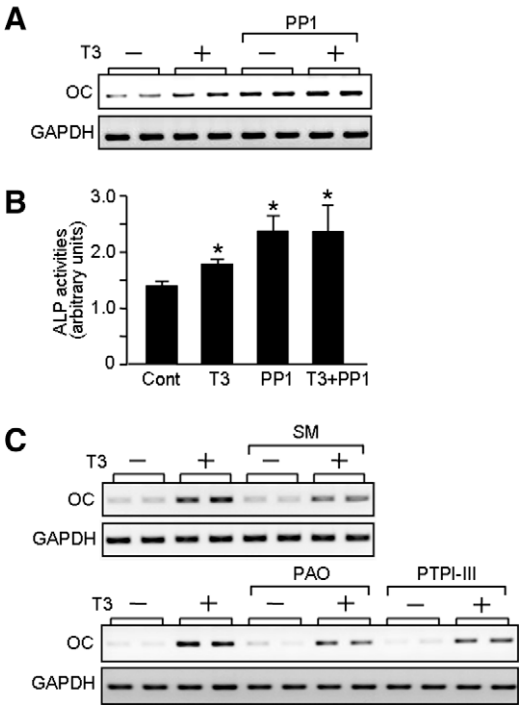

Fig. 3. T3-dependent expression of OC is sensitive to PP1 and PTPI. (A) Osteoblasts were treated for 36 h with 10 nM T3 in the presence and absence of 2 μM PP1. Total RNA was subjected to RT-PCR to analyze the expression of OC and GAPDH. Experiments were performed in duplicate culture. (B) Osteoblasts were treated for 5 days as described above, and the ALP activities were measured using whole cell lysates. ALP activities were normalized by the protein levels, and expressed as arbitrary units. Mean ± SD ($n = 6$). $^*p < 0.05$ vs. the levels in the cells without T3 (control). (C) Osteoblasts were treated for 36 h with 10 nM T3 in the presence and absence of sodium molybdate (SM, 2 μM), phenylarsine oxide (PAO, 20 nM), or protein–tyrosine phosphatase inhibitor III (PTPI-III, 10 μM). Total RNA were subjected to RT-PCR.

Overexpression of SrcY527F attenuates T3-dependent expression of OC

To confirm the contribution of Src to T3-dependent expression of OC, we overexpressed Y527F and WT in primary mouse calvarial osteoblasts. As shown in Fig. 4A, Western blot analysis revealed the increased expression of WT and Y527F in the transfected cells, when compared to the non-transfected cells. T3 had no effects on the protein levels of Src in the transfected cells. The levels of Y527 phosphorylation were also increased in the transfected cells, but T3-dependent alteration was not detected.

In the cells transfected with WT, T3 suppressed the phosphorylation of Y416, and the expression of OC was significantly increased (Fig. 4B). By contrast, in the cells transfected with Y527F, the Y416 phosphorylation was markedly increased in the basal conditions (Fig. 4A). The phosphorylation levels maintained very high even in the presence of T3. T3-dependent increase of OC expression was markedly inhibited in the cells transfected with Y527F

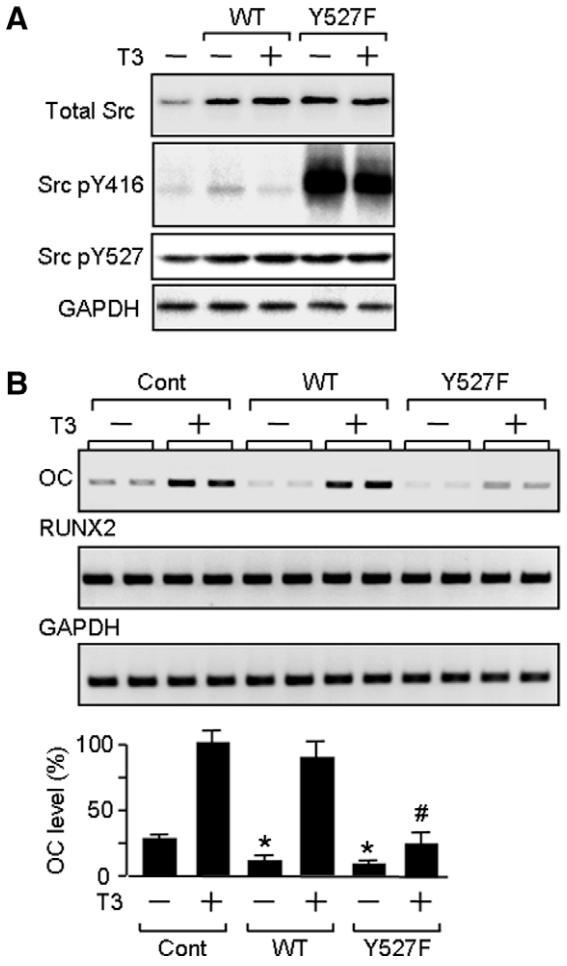

Fig. 4. Effects of overexpression of SrcY527F on T3-dependent expression of OC. (A) Mouse calvarial osteoblasts were transfected with plasmid expressing wildtype Src (WT) or SrcY527F. Whole cell lysates were subjected to Western blot analysis using antibodies against Src, phospho-Src (Y416), phospho-Src (Y527), and GAPDH. (B) Transfected osteoblasts were treated with 10 nM T3 for 36 h, and subjected to RT-PCR. In densitometric analysis, the OC levels were normalized by the GAPDH levels, and expressed as percentage of the levels in the non-transfected cells treated with T3. Mean ± SD ($n = 4$). $^{*}p < 0.05$ vs. the levels in the non-transfected cells without T3. $^{\#}p < 0.05$ vs. the levels in the cells transfected with WT and treated with T3.

(Fig. 4B), indicating that the suppression of Src is required for T3-dependent expression of OC. RUNX2 expression was not affected by the overexpression of WT and Y527F.

Discussion

Several kinases are demonstrated to be regulated by thyroid hormone in a non-genomic manner, which include phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase [17,19–25]. It is also demonstrated that actin polymerization is under control of thyroid hormone through phosphorylation, which plays important roles in the migration of astrocytes and granular neurons [26]. In this study, we provide an additional example, in which T3 rapidly and persistently suppresses the kinase activity of Src in primary calvarial osteoblasts prepared from neonatal mice (Figs. 1 and 2). Since the phosphorylation levels of Y527 were not altered, it is unlikely that the observed effect of T3 is mediated by Y527 kinases or phosphatases [27,28].

The present study also shows that the persistent suppression of Src activity is associated with osteoblast differentiation as evidenced by the increased expression of OC and ALP (Fig. 2). Pharmacological suppression of Src by PP1 also stimulates OC expression and ALP activities (Fig. 3). To confirm the involvement of Src in T3-dependent expression of OC, constitutively active Src (Y527F) was overexpressed in primary mouse calvarial osteoblasts (Fig. 4). In such cells, marked activation of Src was demonstrated, and the suppressive effect of T3 on Src was limited. Accordingly, T3-dependent expression of OC was significantly attenuated. These results indicate that the suppression of Src by T3 induces the expression of OC. On the other hand, although Y527F was very active, T3-dependent OC expression was not completely prevented, suggesting the presence of other pathway(s) leading to the OC expression.

RUNX2 is a key transcription factor stimulating the expression of the OC gene [18], but in this study, no increase of RUNX2 expression was observed in response to T3 (Figs. 2 and 4), suggesting that posttranslational activation of RUNX2 may lead to OC expression. Recently, it was demonstrated that RUNX2 forms a complex with a co-repressor, YAP (Yes-associated protein), that is a target of Src [29]. When Src activity is decreased, the dephosphorylated YAP dissociates from RUNX2, which triggers the transcription of the OC gene by RUNX2.

Interestingly, PTP inhibitors, SM, PAO, and PTPI-III, reversed the decrease of Y416 phosphorylation by T3 (Fig. 1). It was reported that protein–tyrosine phosphatase-BL and low-molecular-weight protein–tyrosine phosphatase *(LMWPTP)* can dephosphorylate phospho-Y416 in a regulatory manner [30,31]. The activity of LMWPTP is increased in response to a rise of intracellular reduced glutathione [31,32]. On the other hand, T3 increases the cellular glutathione levels by enhancing oxidative phosphorylation and production of NADPH [33]. It is thus speculated that the activation of LMWPTP by T3 via a redox mechanism is one of the plausible mechanisms for the decrease of Y416 phosphorylation. In conclusion, the present study demonstrates a novel, non-genomic action of T3 on Src, leading to osteoblast differentiation. We are now investigating the molecular basis as to how T3 suppresses Src.

Acknowledgments

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