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Transcriptional repression of Type I procollagen genes during adipocyte differentiation

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Abbreviations: ECM, extracellular matrix; DEX, dexamethasone; IBMX, isobutylmethylxanthine; ORO, oil red O

Abstract

Adipocyte differentiation is a very complex process in which whole-cell changes are accompanied. Among them, type I procollagen gene has been shown to specifically decrease during adipocyte differentiation; however, little is known about the molecular mechanism. To examine how type I procollagen gene expression is regulated at the level of transcription during adipocyte differentiation, 3T3-L1 preadipocyte cell line was used as an *in vitro* model. Northern blot analysis demonstrated that mRNA expression of type I procollagen gene was dramatically reduced during adipocyte differentiation. Time-course analysis indicated that decrease in mRNA expression occurred at early stage of differentiation. Studies on several stable cell lines showed that transcriptional activities of both α1 and α2 promoters decreased significantly during adipocyte differentiation. Despite extensive deletion-promoter analyses, however, we could not identify the *cis*-element responsible for the switch-off of type I procollagen gene during adipocyte differentiation, suggesting that the transcriptional repression of this gene occur through general transcription machinery rather than a specific *cis*-element. In conclusion, down-regulation of type I procollagen mRNA expression during adipocyte differentiation is due to repression of its promoter activity through general transcription machinery.

Keywords: Procollagen; adipocyte differentiation; transcription; repression

Introduction

Adipocyte differentiation is a very complex process that is triggered and promoted by coordinated signals of growth factors, cytokines, and hormones. Besides significant increases in mRNAs of the genes directly involved in lipidogenesis, changes occur in expression of cytoskeletal, extracellular matrix (ECM), and related components such as actin, tubulin, fibronectin, and collagen (Spiegelman and Farmer, 1982; Antras *et al*., 1989; Weiner *et al*., 1989). Modulation of cytoskeletal and ECM components could allow for cytoskeletal rearrangement, release of cell adhesion, and remodeling of cell components necessary for morphological changes. However, less attention has focused on the modulations in cytoskeletal and ECM components accompanying adipocyte differentiation. During differentiation, expressions of actin, tubulin, fibronectin, type I and type III collagen, and pref-1 decrease whereas expressions of CSPG-1, entactin/nidogen, and type IV collagen increase (Spiegelman and Farmer, 1982; Aratani and Kitagawa, 1988; Antras *et al*., 1989; Weiner *et al*., 1989; Calvo *et al*., 1991; Smas and Sul, 1993). Among these, collagen is the major ECM protein and shown to drastically decrease in mRNA level by 80-90% during 3T3-L1 differentiation (Weiner *et al*., 1989). However, little is known about the molecular mechanism of down-regulation of the collagen gene during adipocyte differentiation. Alteration in transcription of a gene implies that regulatory modulation may occur at the level of its promoter. We studied how type I procollagen gene transcription could be regulated at the level of its promoter during adipocyte differentiation, employing 3T3-L1 mouse preadipocyte cell line as an *in vitro* model system.

Materials and Methods

Cell culture and differentiation

3T3-L1 preadipocytes (ATCC CL-173 CCL-92.1) were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% calf serum in a 37°C humidified incubator. Subconfluent cells were induced to differentiate to adipocytes by feeding with DMEM containing 10% fetal bovine serum (FBS) plus 1 μg/ml of insulin (Sigma, St. Louis, MO), 0.5 mM of isobutylmethylxanthine (IBMX) (Sigma), and 0.25 μM of dexamethasone (DEX) (Sigma). At 48 h postinduction, the cells were re-fed with the fresh medium containing FBS plus 1 μg/ml of insulin only. The cells were cultured for at

least additional 3 days to further differentiate to mature adipocytes.

Oil-Red O (ORO) staining

Lipid accumulation in 3T3-L1 cells was identified by ORO staining. After 3T3-L1 preadipocytes were induced to differentiate into mature adipocytes, they were rinsed with phosphate-buffered saline (PBS), fixed with 70% ethanol for 5 min., and then stained with ORO for 3 min. Stained cells were extensively rinsed with PBS and distilled water. The cells were counter-stained with Harris-hematoxylin for 1 min and then rinsed with distilled water.

Northern blot hybridization

Northern blot hybridization was done as described previously (Rhew *et al.*, 1999). The cDNA probe used for hybridization was HF677, human α1(I)procollagen cDNA (Chu *et al.*, 1992).

Plasmids

pGL2(-)4.0α1(I), and pGL2(-)220α1(I) are both luciferase reporter plasmids containing α1 chain promoter constructs of mouse type I procollagen gene. To create pGL2 (-)4.0α1(I), a 4.2 kb-fragment cut from pJ400 (kindly provided by Dr. Benoit de Crombrugghe, MD Anderson Cancer Center, University of Texas, Houston, TX) by *Bam*HI was inserted into *Bgl* II site of pGL2-Basic (Promega, Madison, WI). A fragment of 336 bp generated by *Hind* III-*Bgl* II digestion from pG70 was subcloned to *Hind* III-*Bgl* II site of pGL2-Basic to produce pGL2(-) 220α1(I). The other reporters containing several collagen promoter constructs were kindly provided by Dr. Benoit de Crombrugghe.

Stable transfection and luciferase assay

All transfections were performed by using LipofectAMINE™ reagent (Life Technologies, Gaithersberg, MD) according to manufacturer's instructions. Cells were cotransfected with several luciferase reporter genes and a neomycin-resistant selection marker gene. At 48 h posttransfection, the cells were subjected to selection with the medium containing 700 μg/ml of G418. The cells were re-fed with selection medium every three days until the colonies were apparently formed. At least more than 25 colonies were obtained from each transfection. The colonies were then pooled and cultured in the selection medium. Luciferase assay was carried out as described previously (Yi *et al.*, 2000).

Results

As an *in vitro* model system for adipocyte differentiation study, a mouse preadipocyte cell line 3T3-L1 was employed. 3T3-L1 cells are easily induced to differentiate to adipocytes by combinatorial mixture of insulin, DEX, and

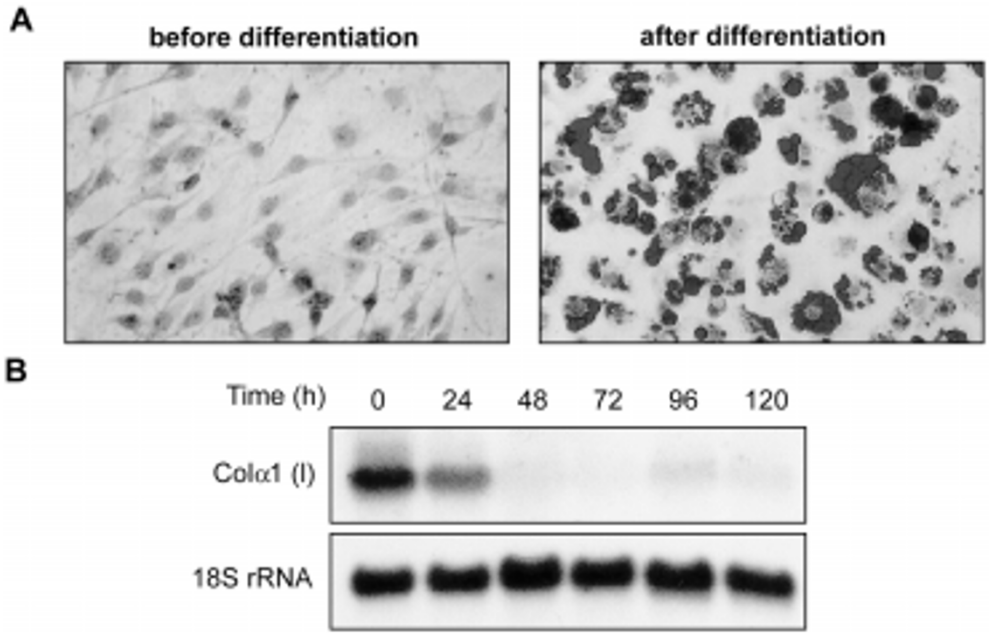

Figure 1. (A) Morphology of 3T3-L1 cells. *left panel.* 3T3-L1 preadipocytes. *right panel.* 3T3-L1 cells after induction to mature adipocytes. Lipid accumulation was visualized by ORO staining. ORO staining showed that no obvious lipid droplet was observed in preadipocytes whereas differentiated cells exhibited round shape characteristic to adipocytes and nearly all of them contained well-developing red-colored lipid droplets (magnitude: ×10). (B) Decrease in mRNA in differentiated 3T3-L1 Cells. Northern hybridization was performed to examine whether mRNA expression is altered during adipocyte differentiation. After induction, 3T3-L1 cells were harvested at the indicated time. Northern blot analysis showed that mRNA level of differentiated 3T3-L1 cells is drastically decreased and that this occurs at early stage of differentiation. *Colα1(I): α1 chain of type I procollagen.*

IBMX. To examine whether 3T3-L1 preadipocytes can be induced to fully differentiate to mature adipocytes in our system, adipocytes after induction were identified with ORO staining which selectively stains lipid-droplets. ORO staining showed that adipocyte differentiation of 3T3-L1 cells was well induced in our system (Figure 1A). Next, it was examined whether mRNA level of type I procollagen would be changed during adipocyte differentiation. Northern blot analysis showed that mRNA level of type I procollagen decreased as early as 24 h after induction and little signal was detected after 48 h (Figure 1B), suggesting that transcription of type I procollagen is down-regulated in the early stage of adipocyte differentiation. Our result of decreased transcription of type I procollagen gene during adipocyte differentiation is well consistent with the previous report (Weiner *et al.*, 1989).

To test whether decreased mRNA level of type I collagen is due to decrease in its promoter activity, reporter plasmids containing type I procollagen promoter constructs were made (Figure 2A). First, 3T3-L1 preadipocytes were transfected with α1 chain promoter, pGL2(-)4.0α1(I), and α2 chain promoter, pH5, along with pH2Rneo, a neomycin-resistant gene-containing vector. Stable transfectants were selected with G418-containing selection medium for several weeks. At least more than 25 colonies were obtained and they were pooled. These stable transfectants were designated as L1pGL2(-)4.0α1(I) and L1pH5, respectively. After induced to mature adipocytes for 5 days, they were harvested and then assayed. Luciferase assay showed that the promoter activities of both cell lines were drastically reduced after differentiation (Figure 2B). Because mRNA level of type I procollagen decreased at early stage of differentiation (Figure 1B), we investigated whether its promoter activity is also suppressed at early stage of differentiation to cause decrease in mRNA expression. L1pH5 was induced to differentiate, harvested at the indicated times, and then assayed. Upon differentiation, the promoter activity decreased even at 24 h (Figure 2C). Consistent with the Northern

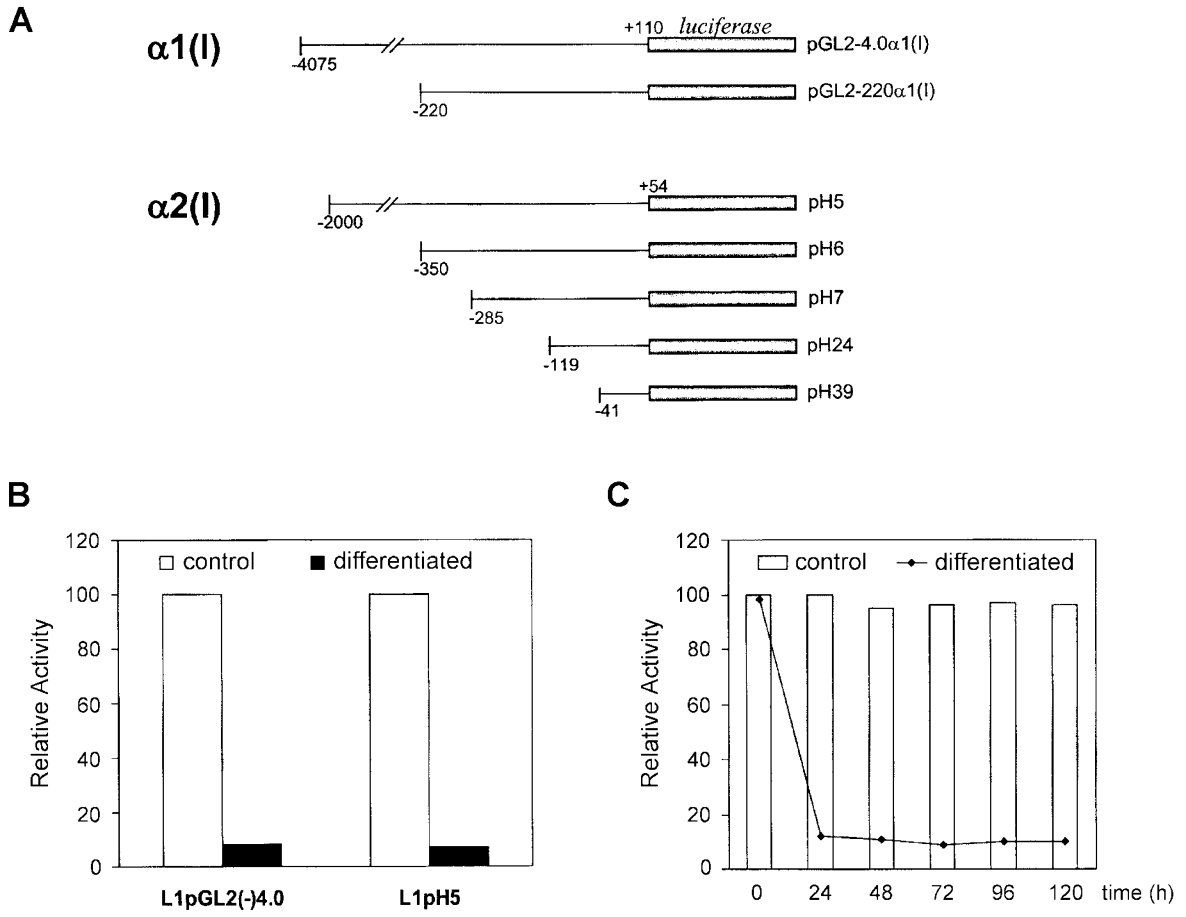

Figure 2. (A) Luciferase reporter genes containing several deletion promoters of type I procollagen. (B) Transrepression of type I procollagen promoters during adipocyte differentiation. At 5 days postinduction, the indicated stable cell lines expressing pGL2(-)4.0 or pH5 were harvested and assayed for promoter acitivities. When differentiated, the promoter activities of both cell lines were drastically reduced. (C) The promoter activity of L1pH5 was significantly reduced within 24 h after induction, which is well consistent with the Northern blot analysis.

blot analysis, these results demonstrated that early down-regulation of type I procollagen mRNA expression is due to its decreased promoter activity at early stage of adipocyte differentiation.

We hypothesized that if the *cis*-element responsible for the switch-off of type I procollagen gene transcription during adipocyte differentiation may reside in its promoter, *cis*-element-truncated mutant promoter would not respond to differentiation signals. To ascertain our hypothesis, a series of sequential deletion mutant promoter constructs was made (Figure 2A). 3T3-L1 preadipocytes were transfected with each of promoter constructs along with pH2Rneo. Stable transfectants were made and selected. L1pGL2(-)220α1(I), and L1pGL2(-)30 were 3T3-L1-derived cell lines stably transfected with type I collagen α1 chain promoter constructs of pGL2(-)220α1(I), and pGL2(-)30α1(I), respectively. L1pH6, L1pH7, L1pH24, and L1pH39 were 3T3-L1-derived cell lines stably transfected with type I collagen α2 chain promoter constructs of pH6, pH7, pH24, and pH39, respectively. Each of these cell lines along with L1pGL2(-)4.0α1(I) and L1pH5 was induced to differentiate to adipocytes. At 5 days post induction, the cells were harvested and assayed for luciferase activity. When differentiated, the promoter activity of pGL2(-)220α1(I) was decreased as much as that of pGL2(-)4.0α1(I) (Figure 3A). Decrease in the promoter activity was also observed in α2 chain reporters-transfected cell lines. The promoter activities of all of constructs from pH5 to pH24 were also reduced significantly (Figure 3B). The activity reduction of L1pH24 was not so much as those of the others. Maybe this is thought to be caused by its lower extent of differentiation, not by de-repression. Result from the shortest pGL2(-)30α1(I) and pH39 containing just the minimal promoter was not shown because their basal activities were nearly the background. Taken together, these data suggest that the *cis*-element may be present within 220 bp of α1 promoter and within 119 bp of α2 promoter of type I procollagen gene.

To examine whether *cis*-element may be present within 119 bp of α2 promoter, we made additional stable cell lines overexpressing internal deletion promoter reporters. pTH13 and pTH5 are luciferase reporters of promoters in which the segment between 130 and 40 bp is internally deleted from pH5 and pH6, respectively (Figure 4A). L1pTH13 and L1pTH5 are 3T3-L1-derived cell lines stably expressing pTH5 and pTH6, respectively. After induced to differentiate to adipocytes, the cells were harvested and assayed for the promoter activity. The promoter activities of differentiated cells were still reduced so much as those of their parental constructs (Figure 4B), suggesting that during adipocyte differentiation, type I procollagen gene may be regulated by a mechanism governing differentiation-dependent transcriptional repression other than a regulatory mechanism exerted by a master *cis*-element.

Discussion

Preadipocyte conversion to fat-rich adipocyte is a very complicated process requiring whole-cell alterations in cellular metabolism, morphology, gene expression, and so on. Although more than 300 proteins were shown to be dramatically altered during 3T3-L1 differentiation, the majority of these have not been characterized yet (Sadowski *et al.*, 1992). The molecular mechanisms governing transcriptional regulation during adipocyte differentiation have been addressed for only a few of these genes. Studies to date have largely focused on transcriptional activation during adipocyte differentiation. More intention has been given to lipidogenesis or lipid metabolism-related cellular changes whereas little is known about the regulation of ECM or cytoskeletal genes of which modulations account for substantial changes in architectural and structural complexity of

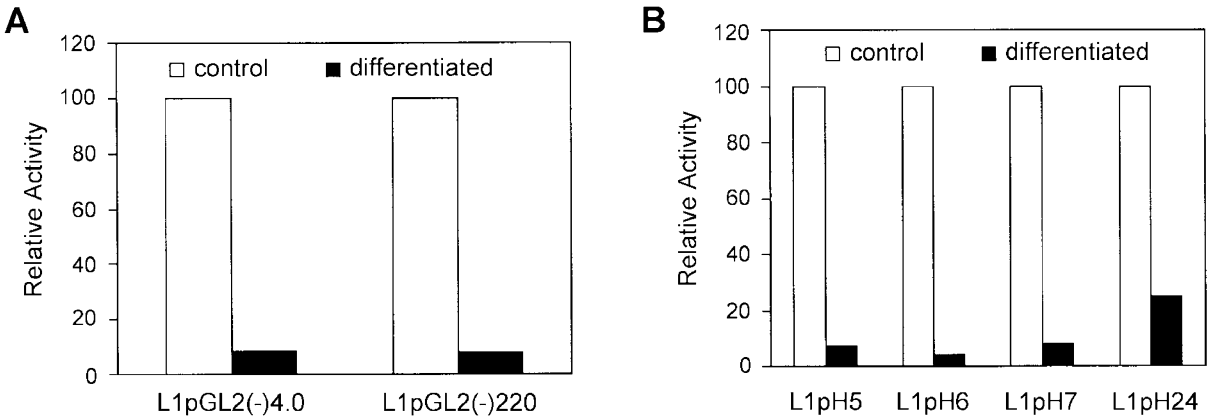

Figure 3. Sequential deletion-promoter analysis of type I procollagen promoters during adipocyte differentiation. (A) Colα1(I). (B) Colα2(I). Each of stable transfectants was induced to differentiate to adipocyte. At 5 days postinduction, the cells were harvested and assayed. When differentiated, the promoter activities of all cell lines were still significantly reduced.

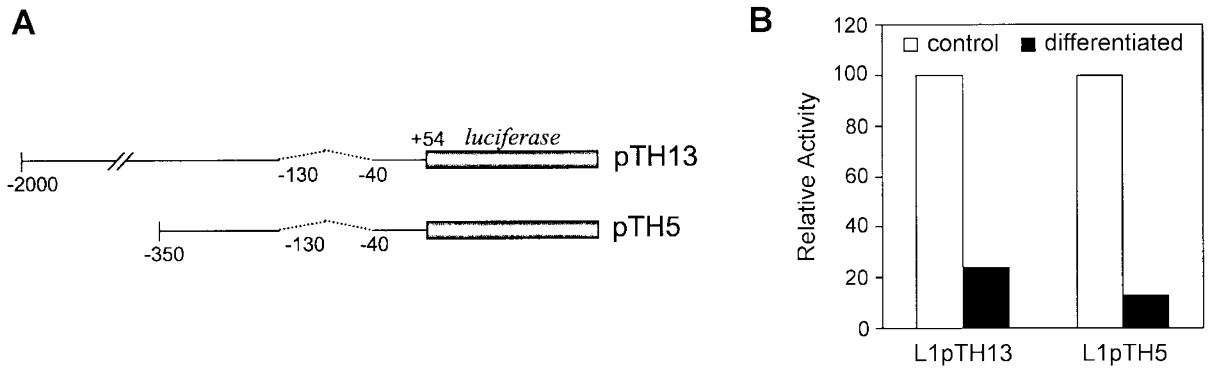

Figure 4. Analysis of internal deletion mutant promoters of Cola2(I) during adipocyte differentiation. (A) Schematic structures of internal deletion promoter constructs of pTH13 and pTH5. (B) Both of stable transfectants was induced to differentiate to adipocyte. At 5 days postinduction, the cells were harvested and assayed. The promoter activities of pTH13 and pTH5 was still observed to decrease so much as their parental constructs, pH5 and pH6, respectively.

adipocytes (Chapman *et al.*, 1984; Bernlohr *et al.*, 1985; Cook *et al.*, 1985). Moreover, genes that are repressed during adipocyte differentiation have been given less attention whereas transcriptional activation of genes has received extensive attention. ECM is not merely a structural or an architectural entity, but plays an important role in transducing signals into cells for various cell behaviors. Collagen is the most abundant component of ECM and serves as substratum for cell adhesion, migration, spreading, and anchorage-dependent growth (Culp *et al.*, 1979; Yamada, 1982; Spiegelman and Ginty, 1983). Prior to differentiation, 3T3-L1 preadipocytes resemble typical fibrobalsts. Once induced to differentiate, cells become thinner and enlarged followed by assuming signet-ring appearance of mature adipocytes. These morphological changes imply alterations in ECM protein expression. Actually, type I procollagen gene was shown to decrease in its mRNA level during adipocyte differentiation, and that was also confirmed in this study. Our study demonstrated that down-regulation of type I procollagen mRNA expression is caused by its decreased promoter activity during adipocyte differentiation. Transcriptional repression of type I procollagen is specific to preadipocyte cells; there was no repression observed in a non-adipogenic fibroblast cell line C3H10T1/2 which was stably transfeced with pH5 when treated with the differentiation-inducing reagents (data not shown). We tried to identify the *cis*-element that governs gene transcription of type I procollagen during adipocyte differentiation. Deletion promoter analysis, however, showed that there is no *cis*-element responsible for the switch on/off of type I procollagen gene.

Repression mechanisms are less understood than activation mechanisms. Generally, transcriptional repression can be categorized as the followings (Herschbach and Johnson, 1993; Johnson, 1995; Hanna-Rose and Hansen, 1996); first, inactivation of an activator causes repression through posttranslational modification of the activator, dimerization of the activator with a nonfunctional partner, competition for the activator's binding site, or a direct interaction between repressor and activator (Benezra *et al.*, 1990; Momand *et al.*, 1992; Munshi *et al.*, 1998). Second, repression can be accomplished by repressor proteins that associate with general transcription factors and thereby inhibit the formation of pre-initiation complex (Orphanides, 1996). Third, repression can be mediated by a specific DNA element and sequence-specific DNA-binding repressor protein (Hanna-Rose and Hansen, 1996). No *cis*-element and nuclear proteins responsible for repression of preadipocyte genes during adipocyte differentiation have been identified except for *pref-1* gene (Smas *et al.*, 1998). In the case of type I procollagen, it is deduced from our data that transcriptional repression may not be mediated by a certain DNA element of the promoter. Rather, repression may occur through general transcription machinery directly modulated by differentiation signals in this gene. Also it is possible that differentiation signals may down-regulate expression of transcription factors responsible for the basal expression of type I procollagen, thereby switching-off the collagen gene transcription. However, these possibilities remain to be investigated.

Several classes of transcription factors have been shown to be important in adipocyte conversion (MacDougald and Lane, 1995; Brun *et al.*, 1996; Fajas *et al.*, 1998). Of these, PPARγ and C/EBPα have been demonstrated as key regulators both in adipocyte differentiation and in maintaining adipocyte phenotype. PPARγ is strongly induced during adipocyte differentiation and activates several adipocyte-specific genes (Spiegelman and Flier, 1996; Mandrup and Lane, 1997). C/EBPα binds to and transactivates the promoters of many genes that are specifically expressed during adipocyte differentiation (Christy *et al.*, 1989; Kaestner *et al.*, 1990). Although these findings revealed that PPAR and C/EBP families are crucial for adipocyte differentiation, it is unlikely that they affect transcriptional repression of type I procollagen gene. Time-course showed that

significant decrease in type I procollagen mRNA was observed within 24 h whereas it has been demonstrated that PPARγ and C/EBPα are induced at later stage and their expressions are maximal at terminal stage (MacDougald and Lane, 1995; Cowherd *et al*., 1999). Actually, transient transfection showed that overexpression of PPARγ and its heterodimer partner RXRα did not affect the promoter activity of type I procollagen gene (data not shown). Therefore, it would be speculated that transcriptional repression of type I procollagen gene is requisite for adipocyte differentiation and not governed by these master regulatory proteins that promotes adipocyte differentiation.

Taken together, this work demonstrates that down-regulation of type I procollagen gene during adipocyte differentiation is apparently caused by repression of its promoter activity and suggests that transcriptional repression may be mediated by general transcription machinery rather than a specific-DNA element on the promoter.

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