



TR4 activates FATP1 gene expression to promote lipid accumulation in 3T3-L1 adipocytes

Hojung Choi^a, Seung-Jin Kim^a, Sung-Soo Park^a, Chawnshang Chang^b, Eungseok Kim^{a,*}

^a Department of Biological Sciences, College of Natural Sciences, Chonnam National University, 300 Yong Bong-Dong, Buk-Gu, Gwangju 500-757, South Korea

^b George Whipple Laboratory for Cancer Research, Departments of Pathology, Urology and Radiation Oncology, and Cancer Center, University of Rochester Medical Center, Rochester, NY, USA

ARTICLE INFO

Article history:

Received 28 April 2011

Revised 25 July 2011

Accepted 3 August 2011

Available online xxxxx

Edited by Robert Barouki

Keywords:

TR4

FATP1

Adipocytes

Fatty acid uptake

Lipid accumulation

ABSTRACT

We show that TR4 facilitates lipid accumulation in 3T3-L1 adipocytes via induction of the FATP1 gene. Further study showed that TR4 transactivated FATP1 5' promoter activity via direct binding to the TR4 responsive element located at the FATP1 5' promoter region. Constitutive overexpression of TR4 in 3T3-L1 adipocytes resulted in increased lipid accumulation, accompanied by an increase in fatty acid uptake. However, small interfering RNA knockdown of FATP1 abolished TR4-enhanced fatty acid uptake. Moreover, microRNA-mediated silencing of TR4 in 3T3-L1 adipocytes drastically reduced basal FATP1 5' promoter activity and FATP1 expression with reduced lipid accumulation. © 2011 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

1. Introduction

Adipocytes are the principal cells for lipid storage and mobilization in response to nutritional demands. Energy intake increases plasma insulin level, which promotes fatty acid (FA) uptake into adipocytes and intracellular lipid accumulation by esterification of fatty acids with glycerol [1]. Unesterified long-chain fatty acids (LCFAs) are transported into mammalian cells predominantly via a protein-mediated mechanism. Several membrane proteins, including the fatty acid transport protein (FATP) family, FA translocase/CD36, and plasma membrane FA-binding protein have been reported to promote cellular uptake of LCFAs [2]. FATP1 belongs to the FATP family; it was the first of this protein family to be identified and is the best characterized in adipocytes [3]. It has been reported that insulin promotes FATP1 translocation from an intracellular compartment to the plasma membrane and concomitantly increases LCFA uptake [1]. Consistently, gain-of-function studies have shown that FATP1 increases FA import into adipocytes [4]. Moreover, loss of FATP1 in mice decreases FA uptake in adipose tissue and skeletal muscle with reduced fat mass, and elevates plasma FA level [3,5].

TR4, a member of the nuclear receptor superfamily, is able to regulate the target gene expression through binding to different AGGTCA direct repeats (DRs) [6]. Interestingly, recent studies showed that TR4-deficient mice showed reduced lipid deposition

in liver and white adipose tissue with decreased expression of hepatic stearoyl-CoA desaturase1 or CD36 [7,8]. TR4 is expressed in several tissues including adipose tissue, liver, and skeletal muscle, which are important for lipid homeostasis, implying that TR4 may also play a key role in lipid homeostasis of adipocytes [9].

Since the molecular mechanisms of adipogenesis have been extensively studied and well characterized in 3T3-L1 cells, we established 3T3-L1 cells constitutively overexpressing or silencing TR4 to determine the TR4 role in lipid accumulation in adipocytes. We demonstrated that TR4 facilitates lipid accumulation in 3T3-L1 adipocytes via increase of FATP1 expression and LCFA uptake.

2. Materials and methods

2.1. Plasmids and reporter gene assay

See Supplementary data.

2.2. Cell culture, stable transfection, and differentiation

See Supplementary data.

2.3. Gel shift assay, chromatin immunoprecipitation (ChIP) assay and Western blotting

See Supplementary data.

* Corresponding author. Fax: +82 62 530 3409.

E-mail address: ekim@chonnam.ac.kr (E. Kim).

2.4. Real-time quantitative RT-PCR (RT-qPCR)

See Supplementary data.

2.5. Knockdown of FATP1 and fatty acid uptake assay

See Supplementary data.

3. Results

3.1. TR4 induces FATP1 expression in 3T3-L1 adipocytes

To study the effect of TR4 on expression of FA transporter genes in adipocytes, we generated 3T3-L1 preadipocytes constitutively expressing TR4 (3T3-L1-TR4). We induced differentiation of 2-day post-confluent 3T3-L1 preadipocytes (designated day 0) by standard protocol and used day 6 adipocytes to examine the effect of TR4 overexpression on expression of various FA transporter genes. By using RT-qPCR and western blotting analyses, we found that TR4 overexpression significantly induced FATP1 mRNA and protein expression with mild induction of CD36 gene (Fig. 1A and B). Induction of FATP1 gene by TR4 overexpression was further confirmed in three other independent stable cells overexpressing TR4 (Supplementary Fig. 1A). Levels of ACSL1 and FATP1 mRNAs and proteins were also slightly increased in 3T3-L1-TR4 adipocytes (Fig. 1A and B). In contrast, mRNA and protein levels of the adipogenic marker gene aP2 was not significantly changed by TR4 overexpression. In differentiating day 2 MEF cells transiently transfected with TR4, FATP1 expression was also induced in parallel with an increase of TR4 (Fig. 1C). Next, we assessed the impact of TR4 overexpression on the levels of FATP1 mRNA at different stages of adipocyte differentiation. FATP1 mRNA levels in 3T3-L1-TR4 cells were low during the first

two days of differentiation, but were still higher than those of control cells (3T3-L1-C) (Fig. 1D). After four days differentiation, FATP1 mRNA levels were drastically increased in both 3T3-L1-TR4 and 3T3-L1-C cells. However, elevated levels of FATP1 mRNA were observed in 3T3-L1-TR4 cells throughout the course of differentiation.

3.2. TR4 induces FATP1 5' promoter activity via binding to the DR1 site located at the FATP1 5' promoter

We next investigated whether TR4 could transcriptionally regulate FATP1 gene expression using a luciferase reporter linked to the FATP1 5' promoter spanning -554 to $+84$ bp (pGL3-FATP1-554) through transient transfection of NIH-3T3 cells. TR4 induced the FATP1 5' promoter activity in a dose-dependent manner (Fig. 2A). In contrast, the TR4 induction effect on FATP1 5' promoter activity was not observed when pGL3-FATP1-440 or pGL3-FATP1-330 was used (Fig. 2B). Sequence analysis of the FATP1 5' promoter region between -554 and -440 bp revealed that one DR1 sequence (GGGGCAAAGGGCA) was located at -470 to -458 bp. To determine TR4 binding to this putative TR4RE (FATP1-TR4RE), we performed a gel shift assay. In vitro translated TR4 protein, but not the mock-translated control lysate, formed a specific complex with 32 P-labeled FATP1-TR4RE (Fig. 2C; lanes 1, 2, 6 and 7). This TR4/FATP1-TR4RE complex was abolished when an excess amount of FATP1-TR4RE, but not mutant FATP1-TR4RE, was added as competitor (Fig. 2C; lanes 3 and 4). Furthermore, addition of anti-TR4 antibody resulted in the supershift of TR4/FATP1-TR4RE complex (Fig. 2C; lane 5). In contrast, normal IgG or anti-RXR α antibody did not supershift TR4/FATP1-TR4RE complex (Fig. 2C; lanes 8 and 9). To further confirm TR4 binding to endogenous FATP1 5' promoter, we performed ChIP assays in NIH-3T3 cells transiently transfected with TR4 expression plasmid. We found specific TR4

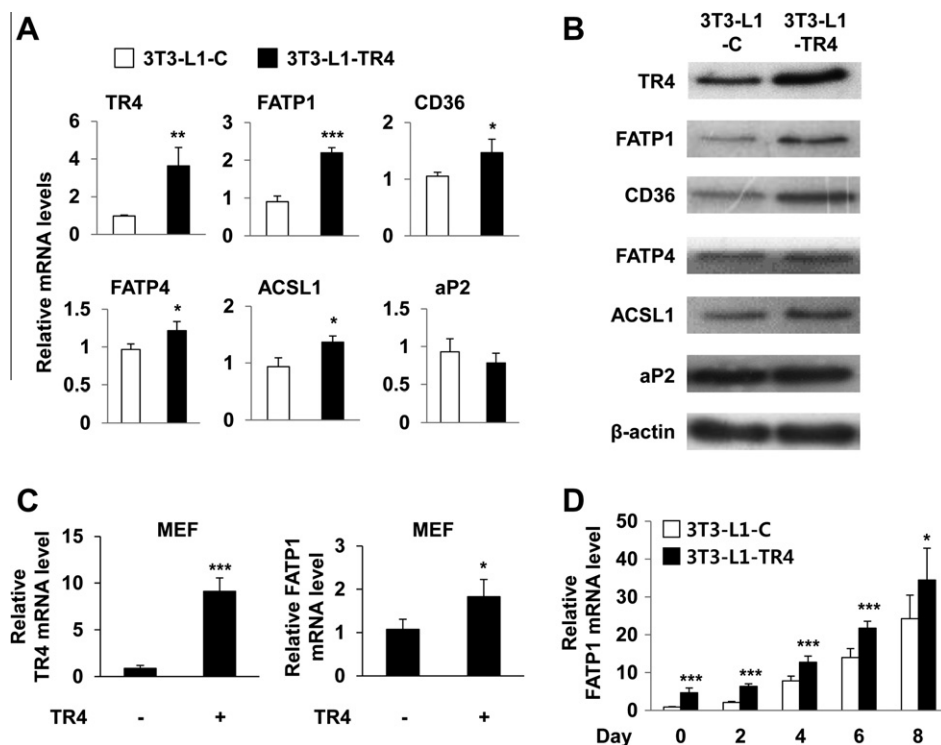


Fig. 1. TR4 increases FATP1 expression in 3T3-L1 adipocytes. (A, B) The mRNA and protein levels of FATP1, CD36, FATP4, ACSL1, and aP2 were analyzed by RT-qPCR and western blotting in TR4 overexpressing (3T3-L1-TR4) or control adipocytes (3T3-L1-C) after six days induction of differentiation (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$). (C) FATP1 mRNA expression in day 2 MEF cells transiently transfected with TR4 expression plasmid or control plasmid was analyzed by RT-qPCR (* $P < 0.05$ and *** $P < 0.001$). (D) FATP1 mRNA expression in differentiating 3T3-L1 adipocytes was analyzed by RT-qPCR (* $P < 0.05$ and *** $P < 0.001$ vs. 3T3-L1-C).

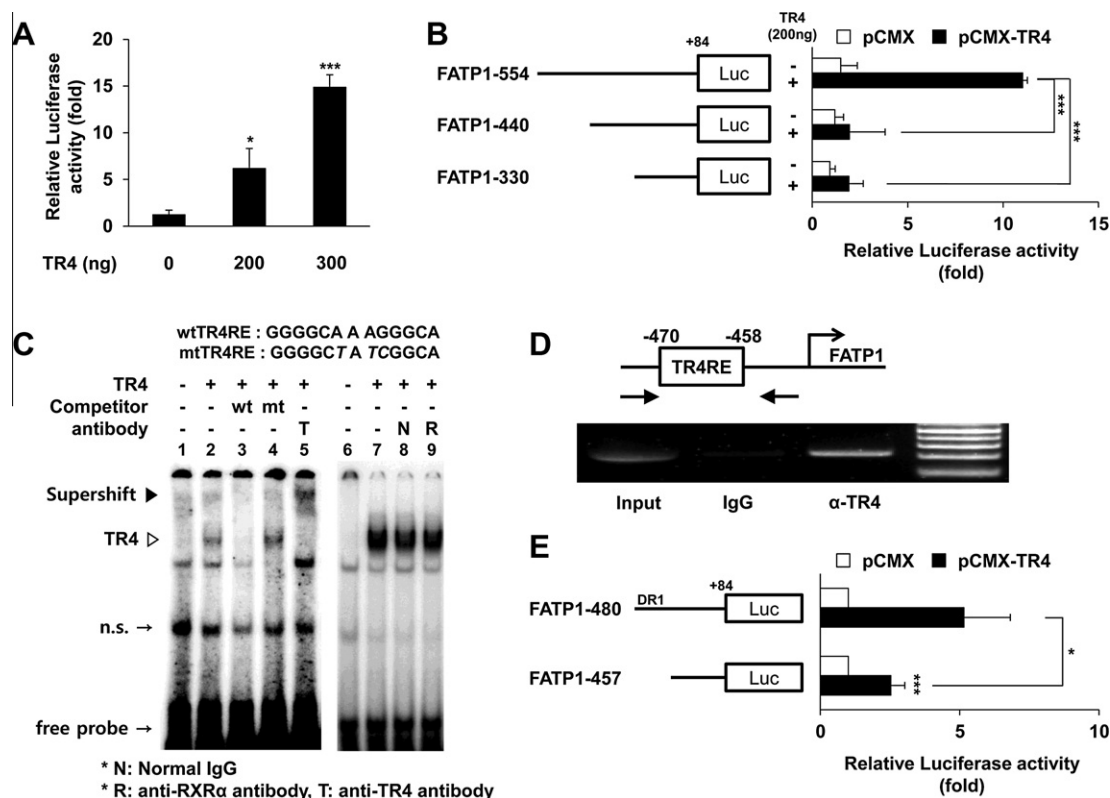


Fig. 2. TR4 promotes FATP1 5' promoter activity via TR4RE. (A, B) Mouse FATP1 5' promoter-fused reporter gene construct (pGL3-FATP1-554, 300 ng) and its deletion constructs (pGL3-FATP1-440 and pGL3-FATP1-330, 300 ng each) were co-transfected with TR4 expression plasmid into NIH-3T3 cells as indicated and luciferase activity was analyzed (* $P < 0.05$ and *** $P < 0.001$). (C) Gel shift assay using 32 P-labeled FATP1-TR4RE. Mock lysate or in vitro-translated TR4 was incubated with 32 P-labeled FATP1-TR4RE. Fifty molar excess of unlabeled wild type (wt) or mutated (mt) oligonucleotides were added as competitors and antibodies were added for supershift assay as indicated. (D) Results of the ChIP assay. The cross-linked protein/DNA complex isolated from NIH-3T3 cells transfected with TR4 expression plasmid was immunoprecipitated with anti-TR4 antibody or normal IgG as indicated. The region (-554 to -341 bp) containing the putative TR4 binding site of the FATP1 5' promoter was amplified from DNA extractions by PCR. Lane 1 contains the input control. (E) Reporter gene construct (250 ng) fused with FATP1 5' promoter either containing (pGL3-FATP1-480) or lacking TR4RE (pGL3-FATP1-457) was co-transfected without or with TR4 expression plasmid (150 ng) into NIH-3T3 cells and luciferase activity was analyzed (* $P < 0.05$ and *** $P < 0.001$ vs. control).

binding to the FATP1 5' promoter region containing FATP1-TR4RE when we performed PCR to amplify the -554 to -341 bp region from protein-DNA complexes immunoprecipitated by anti-TR4 antibody, but not by normal IgG control (Fig. 2D). Next, we analyzed the importance of FATP1-TR4RE on TR4 transactivation of the FATP1 5' promoter using two different reporter plasmids fused to FATP1 5' promoter either containing (pGL3-FATP1-480) or lacking FATP1-TR4RE (pGL3-FATP1-457). When TR4 was co-transfected with pGL3-FATP1-480 into NIH-3T3 cells, TR4 induced the reporter gene activity (Fig. 2E). In contrast, this TR4 induction was dramatically decreased when pGL3-FATP1-480 was replaced by pGL3-FATP1-457. Together, these data strongly suggest that TR4 induces FATP1 gene expression via binding to FATP1-TR4RE located in the FATP1 5' promoter.

3.3. TR4 overexpression increases lipid accumulation in 3T3-L1 adipocytes by enhancing LCFA uptake

Since the function of FATP1 in FA uptake and lipid accumulation in adipocytes is well established, we analyzed the TR4 effect on lipid accumulation in 3T3-L1 adipocytes by using Oil Red O staining. TR4 overexpression increased lipid accumulation in 3T3-L1 adipocytes about 2.8-fold on day 5 and 2-fold on day 9 relative to that of control adipocytes (Fig. 3A) and this TR4-induced lipid accumulation in 3T3-L1 adipocytes was further confirmed in other TR4 over-expressing cells (Supplementary Fig. 1B). Consistently, 3T3-L1-TR4

adipocytes showed about a 2-fold increase of 14 C-labeled oleic acid uptake relative to control 3T3-L1-C adipocytes under insulin-stimulated conditions (Fig. 3B). Knockdown of FATP1 by FATP1-specific siRNAs reduced LCFA uptake in both control and 3T3-L1-TR4 adipocytes. Moreover, this silencing of FATP1 abolished TR4-enhanced LCFA uptake in 3T3-L1-TR4 adipocytes. Knockdown of FATP1 significantly decreased mRNA levels of FATP1, but not of CD36, ACSL1, and FATP4 in 3T3-L1-TR4 adipocytes, suggesting that TR4 facilitates LCFA influx into 3T3-L1 adipocytes via modulation of FATP1 expression (Fig. 3C). In addition, when day 6 adipocytes were treated with 100 μ M oleic acid for 48 h, oleic acid-induced cellular lipid accumulation in 3T3-L1-TR4 adipocytes was more obvious than that of 3T3-L1-C adipocytes (Fig. 3D).

3.4. Silencing of TR4 inhibits FATP1 expression and lipid accumulation in 3T3-L1 adipocytes

To further determine the effect of TR4 on FATP1 expression and lipid accumulation in 3T3-L1 adipocytes, we generated 3T3-L1 preadipocytes stably expressing scrambled (3T3-L1-miR) or three different TR4 microRNAs (3T3-L1-TR4miR1, 3T3-L1-TR4miR2 and 3T3-L1-TR4miR3) (Fig. 4A and Supplementary Fig. 2A). TR4 knockdown by TR4miR3 significantly inhibited mRNA expression of FATP1 gene in day 8 3T3-L1 adipocytes by 60% compared with 3T3-L1-miR adipocytes. However, TR4 and FATP1 expression was

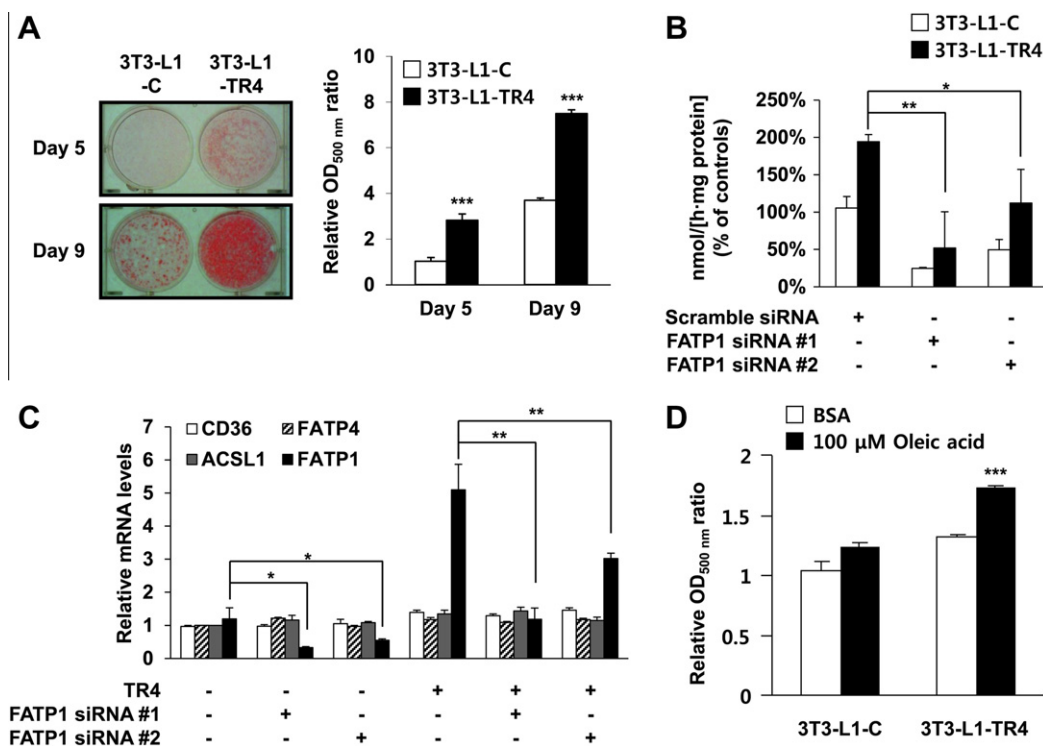


Fig. 3. TR4 promotes lipid accumulation in 3T3-L1 adipocytes via increase of FA uptake. (A) 3T3-L1 preadipocytes (3T3-L1-TR4 and 3T3-L1-C) were differentiated and stained with Oil Red O to determine lipid accumulation on the days indicated ($***P < 0.001$ vs. 3T3-L1-C). (B) After 72 h transfection of FATP1 siRNAs or scrambled siRNA to day 5 adipocytes (3T3-L1-TR4 or 3T3-L1-C), cells were serum-starved for 1 h and then incubated with 6.2 nmol of ^{14}C -labeled oleic acid for 20 min in HBSS buffer containing 5 μg of insulin ($*P < 0.05$ and $**P < 0.01$). (C) Knockdown effect of FATP1 on the expression of CD36, FATP4, and ACSL1 genes was assessed by RT-qPCR ($*P < 0.05$ and $**P < 0.01$). (D) After six days induction of differentiation, cells were treated with 100 μM oleic acid for 48 h and then stained with Oil Red O to determine oleic acid incorporation into cellular lipids ($***P < 0.001$ vs. 3T3-L1-TR4 cells treated with BSA).

marginally reduced in 3T3-L1-TR4miR1 and 3T3-L1-TR4miR2 adipocytes. CD36 mRNA expression was also reduced in 3T3-L1-TR4miR3 adipocytes although reduction of CD36 mRNA was less significant than that of FATP1 gene. In addition, mRNA levels of aP2 and FATP4 in 3T3-L1-TR4miR3 adipocytes were also mildly reduced compared with 3T3-L1-miR adipocytes. In contrast, ACSL1 mRNA expression was not changed by TR4 knockdown. Western blotting analysis showed that silencing of TR4 resulted in decrease of FATP1 and CD36 protein expression without a significant change in FATP4, ACSL1 and aP2 protein expression (Fig. 4B). Furthermore, silencing of endogenous TR4 in NIH-3T3 cells by transfection of TR4miR3 inhibited basal transcriptional activity of the pGL3-FATP1-554 in a dose-dependent manner (Fig. 4C). Consistently, Oil Red O staining assay revealed that knockdown of TR4 by TR4miR3 resulted in decrease of lipid accumulation in 3T3-L1 adipocytes by 50% relative to the control adipocytes (Fig. 4D and Supplementary Fig. 2C). However, lipid accumulation in 3T3-L1-TR4miR1 and 3T3-L1-TR4miR2 adipocytes was less significant than that of 3T3-L1-TR4miR3 adipocytes.

4. Discussion

Loss of TR4 leads to reduced lipid accumulation in liver and adipose tissues [7,8]. Although TR4 may play important roles in energy homeostasis, the role of TR4 in adipocyte biology remains unknown. In the present study, we demonstrated a key role of TR4 in lipid accumulation in 3T3-L1 adipocytes through the regulation of FATP1 expression and FA uptake. We further showed that TR4 induced FATP1 5' promoter activity via direct binding to the TR4RE located in the FATP1 5' promoter. FATP1 and CD36 promote

LCFA uptake into differentiating adipocytes [2,10]. Interestingly, the expression of CD36 can be regulated by TR4 in macrophages and liver cells [8,11]. Accordingly, levels of CD36 mRNA and protein in 3T3-L1 adipocytes were increased by TR4. In addition, ACSL1 and FATP4 also could be induced in 3T3-L1 adipocytes by TR4. Thus, it is possible that CD36, ACSL1, and FATP4 genes also play a role in TR4-induced lipid accumulation in adipocytes. However, in our study, addition of FATP1 siRNAs into TR4 overexpressing 3T3-L1 adipocytes reduced only FATP1 mRNA level without change in mRNA levels of CD36, ACSL1, and FATP4 and abolished the promoting effect of TR4 on ^{14}C -labeled oleic acid uptake. This strongly suggests that TR4 may facilitate FA uptake into 3T3-L1 adipocytes predominantly via regulation of FATP1, although partial contribution of other genes associated with FA transport in this process could not be ruled out. Furthermore, the TR4 effect on FATP1 expression and lipid accumulation in 3T3-L1 adipocytes were further confirmed by TR4 knockdown, which caused a decrease of FATP1 expression and lipid accumulation. FATP1 transcription is regulated by nutrients and adipogenic regulators. FATP1 mRNA expression is positively regulated by PPAR γ which plays an important role in adipogenesis [12]. Interestingly, the adipogenic hormone insulin negatively regulates FATP1 mRNA expression but does not affect FATP1 protein level in adipocytes [1]. Insulin increases LCFA uptake via promoting FATP1 translocation from an intracellular compartment to the plasma membrane [1]. Thus, this strongly suggests that TR4 and other adipogenic key regulators might orchestrate to fine-tune FATP1 expression under various metabolic cues. Taken together, our study demonstrates that TR4 plays an important role in lipid accumulation in adipocytes via regulation of FATP1 expression.

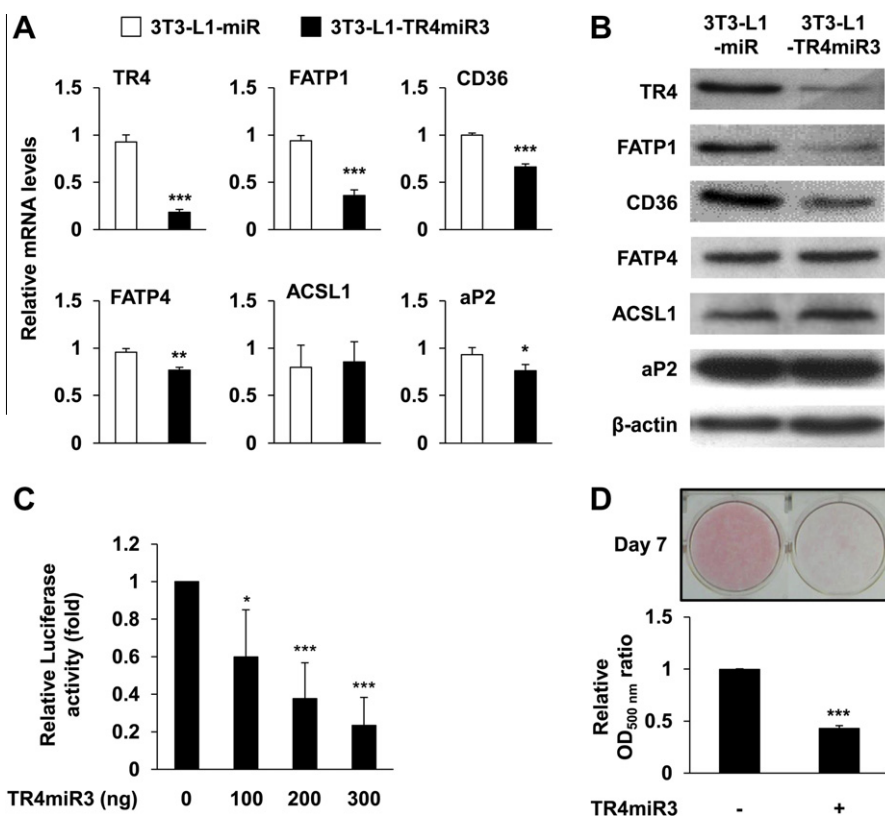


Fig. 4. Silencing of TR4 inhibits FATP1 expression and lipid accumulation in 3T3-L1 adipocytes. (A, B) The mRNA and protein levels of FATP1, CD36, FATP4, ACSL1, and aP2 were analyzed by RT-qPCR and western blotting in TR4 knockdown (3T3-L1-TR4miR3) or control adipocytes (3T3-L1-miR) after six days induction of differentiation ($^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. 3T3-L1-miR). (C) Increasing amounts of TR4miR3 expression plasmid were co-transfected with pGL3-FATP1-554 into NIH-3T3 cells and luciferase activity was analyzed ($^*P < 0.05$ and $^{***}P < 0.001$). (D) Reduction in lipid accumulation of 3T3-L1 adipocytes by TR4 knockdown. Cells were differentiated for seven days and then lipid accumulation was determined by Oil Red O staining ($^{***}P < 0.001$).

Acknowledgements

This work was supported by National Research Foundation of Korea Grant funded by the Korean Government (KRF-2006-312-C00392).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.febslet.2011.08.002](https://doi.org/10.1016/j.febslet.2011.08.002).

References

- [1] Stahl, A., Evans, J.G., Pattel, S., Hirsch, D. and Lodish, H.F. (2002) Insulin causes fatty acid transport protein translocation and enhanced fatty acid uptake in adipocytes. *Dev. Cell* 2, 477–488.
- [2] Pohl, J., Ring, A., Hermann, T. and Stremmel, W. (2004) Role of FATP in parenchymal cell fatty acid uptake. *Biochim. Biophys. Acta* 1686, 1–6.
- [3] Wu, Q., Ortegon, A.M., Tsang, B., Doege, H., Feingold, K.R. and Stahl, A. (2006) FATP1 is an insulin-sensitive fatty acid transporter involved in diet-induced obesity. *Mol. Cell Biol.* 26, 3455–3467.
- [4] Schaffer, J.E. and Lodish, H.F. (1994) Expression cloning and characterization of a novel adipocyte long chain fatty acid transport protein. *Cell* 79, 427–436.
- [5] Kim, J.K. et al. (2004) Inactivation of fatty acid transport protein 1 prevents fat-induced insulin resistance in skeletal muscle. *J. Clin. Invest.* 113, 756–763.
- [6] Kim, E. et al. (2003) Disruption of TR4 orphan nuclear receptor reduces the expression of liver apolipoprotein E/C-I/C-II gene cluster. *J. Biol. Chem.* 278, 46919–46926.
- [7] Kim, E., Liu, N.C., Yu, I.C., Lin, H.Y., Lee, Y.F., Sparks, J.D., Chen, L.M. and Chang, C. (2011) Metformin inhibits nuclear receptor TR4-mediated hepatic stearyl-CoA desaturase 1 gene expression with altered insulin sensitivity. *Diabetes* 60, 1493–1503.
- [8] Kang, H.S. et al. (2011) Nuclear orphan receptor TAK1/TR4-deficient mice are protected against obesity-linked inflammation, hepatic steatosis, and insulin resistance. *Diabetes* 60, 177–188.
- [9] Bookout, A.L., Jeong, Y., Downes, M., Yu, R.T., Evans, R.M. and Mangelsdorf, D.J. (2006) Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network. *Cell* 126, 789–799.
- [10] Kontrova, K. et al. (2007) CD36 regulates fatty acid composition and sensitivity to insulin in 3T3-L1 adipocytes. *Physiol. Res.* 56, 493–496.
- [11] Xie, S. et al. (2009) TR4 nuclear receptor functions as a fatty acid sensor to modulate CD36 expression and foam cell formation. *Proc. Natl. Acad. Sci. USA* 106, 13353–13358.
- [12] Frohnert, B.I., Hui, T.Y. and Bernlohr, D.A. (1999) Identification of a functional peroxisome proliferator-responsive element in the murine fatty acid transport protein gene. *J. Biol. Chem.* 274, 3970–3977.