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Characterization of miR-210 in 3T3-L1 Adipogenesis

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ABSTRACT

Although accumulating evidences indicate that miRNA emerge as a vital player in cell growth, development, and differentiation, how they contribute to the process of adipocyte differentiation remains elusive. In the present study, we revealed that the expression level of miR-210 was dramatically upregulated during 3T3-L1 adipogenesis. Ectopic introduction of miR-210 into 3T3-L1 cells promoted terminal differentiation as well as the expression of adipogenic markers. MTT assay showed that miR-210 significantly inhibited cell proliferation whereas the BrdU incorporation assay and flow cytometry analysis showed that miR-210 did not impair G1/S phase transition. Further experiments demonstrated that enhanced expression of miR-210 in 3T3-L1 cells provoked adipocyte differentiation *via* activation of PI3K/Akt pathway by targeting *SHIP1*, a negative regulator of PI3K/Akt pathway. Moreover, blockade of endogenous miR-210 during adipogenesis significantly repressed adipocyte differentiation. In summary, we have identified miR-210 as an important positive regulator in adipocyte differentiation through the activation of PI3K/Akt pathway. J. Cell. Biochem. 114: 2699–2707, 2013. © 2013 Wiley Periodicals, Inc.

KEY WORDS: MICRORNA; MIR-210; 3T3-L1; ADIPOGENESIS

The mouse 3T3-L1 cell line was the most studied and best characterized *in vitro* model in dissecting the molecular mechanisms of adipogenesis [Green and Kehinde, 1975]. After reaching 100% confluence, the cells initiate growth arrest because of contact inhibition. In the presence of appropriate stimulus such as the standard MDI cocktail containing dexamethasone, insulin, and 3-isobutyl-1-methylxanthine (IBMX), the fibroblast-like 3T3-L1 cells would undergo the transition into round adipocytes. A variety of transcription factors have been identified to contribute to this process, including peroxisome proliferator-activated receptor-γ (PPARγ), CCAAT enhancer-binding protein-α (C/EBPα), C/EBPβ, and C/EBPδ [Rosen et al., 2000; MacDougald and Mandrup, 2002; Rosen and MacDougald, 2006].

Previous studies demonstrated that PI3K/Akt pathway was essential for adipocyte differentiation. Akt1/Akt2 double-knockout mice exhibited reduced capacity to differentiate into mature adipocytes [Peng et al., 2003; Baudry et al., 2006; Yun et al., 2008], and further investigations indicated that both Akt1 and Akt2 were required for the induction of peroxisome proliferator-activated receptor-γ (PPARγ), the key regulator of adipogenesis. Furthermore, constitutively active Akt phosphorylation could provoke 3T3-L1 adipogenesis through relocating the Glucose transporter type 4 (GLUT4) to the plasma membrane and thus increased glucose uptake and lipid synthesis [Kohn et al., 1996; Xu and Liao, 2004]. These evidences indicated that Akt acted as an important player in adipogenesis, however, its upstream regulatory pathways such as miRNA regulation remained largely unknown.

MiRNAs, generally 20–25 nucleotides in length, function by binding to the target mRNA at the post-transcriptional level and hence control cellular mechanisms including embryonic development, organ morphology, cell proliferation and differentiation. The first study [Esau et al., 2004] on human preadipocytes verified that miR-143 served as a positive regulator in adipocyte differentiation. Later, several groups examined the miRNA expression profiles during 3T3-L1 adipogenesis and identified some miRNAs as novel players in 3T3-L1 adipocyte differentiation. Kajimoto et al. [2006] compared the miRNA expression pattern between preadipocytes and mature adipocytes and multiple miRNAs were found to be modulated in fully differentiated adipocytes. Previous studies further demonstrated that increased expression of miR-103 and miR-143 could provoke adipogenesis [Esau et al., 2004; Trajkovski et al., 2011], while increased expression of let-7 and miR-27a/b could suppress 3T3-L1

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adipogenesis [Karbiener et al., 2009; Lin et al., 2009; Sun et al., 2009; Kim et al., 2010]. Recently, miRNA microarrays verified that miR-210 was significantly up-regulated during 3T3-L1 adipogenesis [Sun et al., 2009; Qin et al., 2010], suggesting that miR-210 could be an important regulator in regulating adipogenesis. However, whether miR-210 could target other genes and hence modulate other pathways remains largely unknown. Thus, investigations on the functional role of miR-210 in adipogenesis are lagging behind and the identification of novel miR-210 targets is in demand.

In the current study, we attempted to uncover the role of miR-210 in adipogenic differentiation. We found that expression of miR-210 was up-regulated during 3T3-L1 adipogenesis, and ectopic introduction of miR-210 resulted in increased expression of specific adipogenic markers. Further experiments validated that miR-210 could promote 3T3-L1 adipogenesis through activation of PI3K/Akt pathway *via* targeting SHIP1, a negative regulator in PI3K/Akt pathway.

MATERIALS AND METHODS

3T3-L1 CELL CULTURES AND DIFFERENTIATION

The 3T3-L1 pre-adipocyte and HEK293 cell line were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco BRL) plus penicillin and streptomycin (Gibco BRL). To initiate 3T3-L1 adipocyte differentiation, the classical MDI cocktail (insulin, dexamethasone and 3-isobutyl-1-methylxanthine, all purchased from Sigma) was added to the medium after 2 days post-confluence. After 2 days, the medium was replaced with fresh complete medium containing insulin. Culture medium was changed every 4 days.

OIL RED O STAINING

3T3-L1 cells were seeded into 6 well plates and transfected with miR-210 mimics and negative control. Two days post-confluence (Day 0), the cells were stimulated to differentiate using MDI cocktail. All the samples were collected at Day 6. Briefly, the culture medium was removed and the cells were washed with PBS. The cells were fixed in 4% formalin in PBS for 30 min. The formalin was removed and the fixed cells were washed with PBS thrice. The cells were stained with 0.5% Oil Red O for 20 min. After staining, the cells were washed with PBS and stained with hematoxylin for 10 s and then photographed. To quantify the degree of lipid accumulation, the cells was incubated with isopropanol to extract the lipid droplet and then the Oil Red O staining intensity was measured at the wavelength of 510 nm by a microplate reader (Bio-Rad).

TRANSFECTION ASSAY

For adipocyte differentiation, 3T3-L1 cells were seeded onto six well plates and transfected with miRNA duplex using Lipofectamine™ 2000 (Invitrogen) at a final concentration of 50 nM (miRNA mimics) and 100 nM (miRNA inhibitor) according to the manufacturer's instructions. To increase the transfection efficiency, transfection was repeated three times every 2 days (on Day −2, 0, 2).

For luciferase reporter assay, the miRNA duplex cotransfected with pMIR-REPORT luciferase vector (Ambion) or pMIR-REPORT luciferase vector containing the full length 3′UTR of mouse SHIP1 gene (Addgene) was transfected into the HEK293 cells using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions.

QUANTITATIVE REAL-TIME PCR

Total RNA from 3T3-L1 cells was extracted by Trizol reagent (Invitrogen) and reversely transcribed using QuaniTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. Quantitation of mRNA level by qRT-PCR was performed using ABI 7900HT sequence detection system and the Fast start Universal SYBR Green Master (Roche). The following primers were used for qRT-PCR [O'Connell et al., 2009; Lin et al., 2009; Sun et al., 2009; Kim et al., 2010]: C/EBPα, GACATCAGCGCCTACATCGA (forward) and TCGGCTGTGCTGGAAGAG (reverse); C/EBPβ, ATTTCTATGAGAAAAGAGGCGTATGT (forward) and AAATGTCTTCACTTTAATGCTCGAA (reverse); PPARγ, CAAGAATACCAAAGTGCGATCAA (forward) and GAGCTGGGTCTTTTCAGAATAATAAG (reverse); adiponectin, TGTTCCTCTTAATCCTGCCCA (forward) and CCAACCTGCACAAGTTCCCTT (reverse); aP2, CAAAATGTGTGATGCCTTTGTG (forward) and CTCTTCCTTTGGCTCATGCC (reverse); KLF4, GTGCCCCGACTAACCGTTG (forward) and GTCGTTGAACTCCTCGGTCT (reverse); SHIP1, CCAGGGCAAGATGAGGGAGA (forward) and GGACCTCGGTTGGCAATGTA (reverse); GAPDH, AGGTCGGTGTGAACGGATTTG (forward) and TGTAGACCATGTAGTTGAGGTCA (reverse). The expression of miR-210 during adipogenesis was detected using miScript SYBR Green PCR Kit (Qiagen) with the manufacturer provided miScript Universal primer. Samples were normalized by RNU19 expression levels. The miRNA-specific primer sequences for qRT-PCR were: RNU19, GCTCCAAGCACTGTTTTGGT (forward); miR-210, CTGTGCGTGTGACAGCGGCTGA (forward).

CELL PROLIFERATION ASSAY

The colorimetric MTT assays were performed to determine the cell proliferation. Briefly, 3T3-L1 cells were transfected with control oligo, miR-210 mimics or anti-miR-210 oligo. Twenty-four hours post-transfection, 3T3-L1 cells were seeded into 96 well plates. MTT reagent [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma] was pipetted into each well and incubated at 37°C for 3 h. Then, DMSO was added to dissolve the formazan crystal product at 37°C. The absorbance was measured by microplate reader (Bio-Rad) at the wavelength of 570 nm (690 nm as a reference). All the experiments were performed in tetraplicate and the average reading was calculated.

BrdU INCORPORATION ASSAY

DNA synthesis was measured using the Cell proliferation ELISA BrdU colorimetric kit (Roche) following the manufacturer's protocol. Briefly, 3T3-L1 cells were transfected with miR-210 mimics or negative control and cultured in a 96-well plate. Twenty-four hours later, BrdU was added to the culture medium. After BrdU labeling for 2 h, the medium was removed and BrdU incorporation was measured with the monoclonal anti-BrdU antibody conjugated with peroxidase and tetramethylbenzidine as its substrate. The absorbance was measured at 450 nm.

CELL CYCLE ANALYSIS

3T3-L1 cells transfected with miR-210 mimics or negative control were cultured in six-well plates. Twenty-four hours later, cells were

washed with PBS and harvested. Cells were fixed with 70% ethanol at 4°C overnight, and then stained with staining buffer (50 μg PI/ml, 50 μg RNAse/ml, 0.37 mg/ml EDTA, 1% Triton X-100) at 4°C for 30 min. After the staining, the samples were subjected to cell cycle analysis using LSR Fortessa and FACSDiva software (BD Biosciences).

WESTERN BLOT ANALYSIS

Protein samples from 3T3-L1 cells transfected with miR-210 mimics or negative control were extracted with RIPA lysis buffer. Cellular protein was separated by 10% SDS-PAGE and then transferred to a PVDF membrane. The membrane was incubated with the following primary antibody: total Akt (Cell Signaling Technology), and pAkt (Ser473, BioVision), β-actin (Sigma). Immunoblots were exposed on Super RX X-ray film (Fujifilm).

LUCIFERASE REPORTER ASSAY

Transfection of miRNA duplex with luciferase vector was performed using Lipofectamine™ 2000 (Invitrogen) as previously described. Samples were harvested 48 h after transfection and the firefly luciferase activity was measured by mixing protein extract with luciferase assay buffer using Envision Machine (Wallac). The luminescence levels were normalized to the β-galactosidase activity from pMIR-REPORT Beta-gal vector (Ambion). The β-galactosidase activity was measured by microplate reader (Bio-Rad) at the wavelength of 415 nm.

STATISTICAL DATA ANALYSIS

Data are presented as the means ± S.E.M. for all groups. For analysis on quantitative real-time PCR, the relative expression was normalized to house keeping gene and calculated using the comparative C_T method of $2^{-\triangle\triangle CT}$. Comparisons between two groups were calculated *via* Student's independent t test. Differences were considered to be statistically significant if P values < 0.05.

RESULTS

EXPRESSION PROFILES OF miR-210 DURING ADIPOGENIC DIFFERENTIATION

To test whether miR-210 played a role in 3T3-L1 adipogenesis, qRT-PCR was performed to detect the miR-210 expression profile during adipogenesis. 3T3-L1 adipogenesis was induced using a standard cocktail including IBMX, dexamethasone and insulin on 2 days post-confluence. RNAs were collected at different time points during adipogenesis (Day 0, 2, 4, 6, 8, and 10) and then the expression pattern of miR-210 was determined by qRT-PCR. The expression of miR-210 was found to increase more than twofold during 3T3-L1 differentiation and maintain at a high level until day 10 compared with that of the preadipocyte at Day 0 (Fig. 1A,B), in agreement with previous microarray analysis [Sun et al., 2009], suggesting that miR-210 may play a role in adipogenic differentiation.

To verify the function of miR-210 in adipocyte differentiation, miR-210 mimics or negative control were transfected into 3T3-L1 cells three times every 2 days. The transfection efficiency was monitored by the observation of uptake of a fluorescence labeled small RNA. Further quantitative real-time PCR analysis verified a fivefold increase of mature miR-210 expression at 6 day post-confluence (data not shown). At day 6 post-confluence, a remarkable increase of lipid droplets accumulation was observed in miR-210-transfected 3T3-L1 cells compared with that of cells transfected with negative control which has minimal sequence identity compared with that of the miR-210 of human, mouse, and rat (Fig. 1C,D). Taken together, these data suggest a potential critical role of miR-210 in regulating adipogenesis.

EXPRESSION OF ADIPOGENIC MARKER INCREASES DURING ADIPOGENESIS

In order to elucidate the underlying mechanisms how miR-210 provoked adipogenic differentiation, we investigated the effect of miR-210 on the expression of the well-defined key transcription factors that regulate adipogenic differentiation, such as PPARγ, C/EBPα, C/EBPβ, and KLF4. Their respective mRNA levels were determined in 3T3-L1 cells on day 1 and day 3 during differentiation. On day 1, the expression of most adipogenic marker genes were unaffected by miR-210 (Fig. 2A, C, and E) whereas KLF4 and C/EBPβ showed a significant increase within the first day of hormone induction (Fig. 2B,D). On day 3, almost all adipogenic marker genes increased more than twofold by transfection with miR-210 mimics. However, aP2, a carrier protein for fatty acids that was exclusively expressed in adipocytes and macrophages, was not significantly altered by miR-210 on either day 1 or day 3 as compared with that of control groups (Fig. 2F). Nevertheless, these observations represent the first evidence that miR-210 induces the expression of a class of adipogenic marker genes and hence has the potential to positively regulate adipocyte differentiation.

ENHANCED EXPRESSION OF miR-210 IMPAIRS CLONAL EXPANSION OF 3T3-L1 CELLS

Major identified landmarks of converting preadipocyte into mature adipocyte involve a series of tightly regulated events including growth arrest, post-confluent mitosis, and clonal expansion [Avram et al., 2007]. Since miR-210 was reported to control transition from G1 phase to S phase in specific cancer cells [Tsuchiya et al., 2011; He et al., 2012], we postulated that its overexpression might play an analogous role in modulating adipogenesis. To test this hypothesis, 3T3-L1 cells were transfected with either miR-210 or control oligonucleotides and then subjected to MTT assays. The cell proliferation assay showed a significant retardation of cell growth in 3T3-L1 cells after transfection with miR-210 (Fig. 3A). Then, the effect of inhibition of endogenous miR-210 on cell proliferation was determined using MTT assays. The effect of miR-210 inhibitor on endogenous level of miR-210 was confirmed by qRT-PCR (data not shown). Conversely, inhibition of endogenous miR-210 restored cell proliferation rate (Fig. 3B). In view of aforementioned growth-inhibitory effects of miR-210, we next examined the cell proliferation by 5-bromodeoxyuridine (BrdU) incorporation assays. However, the BrdU assay did not reveal significant change on DNA synthesis after transfection with miR-210 ($P = 0.67$, Fig. 3C).

As we aim to further elucidate the function of miR-210 on cell cycle, flow cytometric analysis was performed. Based on the flow cytometric results, overexpression of miR-210 slightly increased the fraction of cells in the G0/G1 phase and reduced the cells in S phase

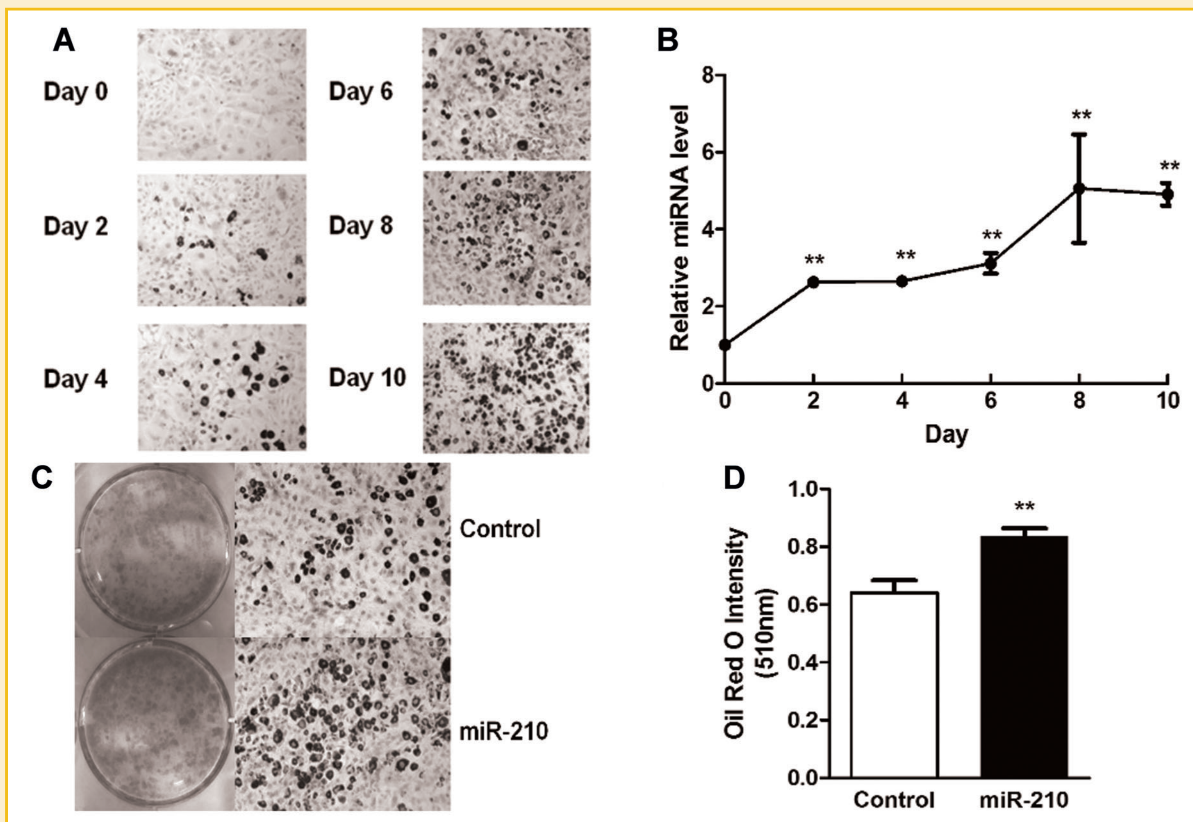

Fig. 1. Overexpression of miR-210 provoked 3T3-L1 adipogenesis. A: Representative microscopic images of lipid accumulation were taken during 3T3-L1 adipogenesis on the days shown (magnification, 200×). B: The expression profiles of miR-210 during adipogenesis were analyzed by qRT-PCR at the indicated time points. The expression of miR-210 was normalized to RNU19 gene (n = 3; $^{**}P < 0.01$ vs. Day 0). C: The effect of miR-210 in 3T3-L1 adipocyte differentiation was determined by Oil Red O staining (magnification, 200×). D: The amount of Oil Red O was quantified after extraction with isopropanol by reading at 510 nm (n = 3; $^{**}P < 0.01$).

(Fig. 3D). Nevertheless, the statistics analysis showed that the corresponding P value of 0.15 was not significant. Since there was no more extra space for cell growth and propagation after reaching 100% confluence, proliferating preadipocytes would not duplicate due to extracellular contact inhibition. Hence, the cell cycle exit of preadipocytes or growth arrest was inevitably a pivotal step in the conversion from preadipocytes towards mature adipocytes [Avram et al., 2007]. According to the flow cytometry and BrdU incorporation assay, miR-210 was showed to suppress cell growth but did not inhibit G1/S phase transition. Taken together, these data supported a potential suppressive role for miR-210 in blocking clonal expansion, which was essential for the conversion from preadipocytes towards mature adipocyte.

MiR-210 ACTIVATES AKT PHOSPHORYLATION

Phosphorylated Akt regulated a large number of substrates involved in diverse biological processes, many of which favored the process of adipocyte differentiation. To investigate the functional role of miR-210 in coordinating Akt pathway and adipogenesis, western blot and rescue experiments were conducted. According to the western blot results, miR-210 was shown to increase the Akt phosphorylation markedly compared with that of the control group (Fig. 4A). Next, we performed a rescue experiment using the PI3K specific inhibitor LY294002 and Wortmannin to investigate whether the activity of PI3K was also required for the miR-210-induced adipogenesis and we verified that the concentration of LY294002 and Wortmannin used in the following experiments did suppress 3T3-L1 adipogenesis (Fig. 4B, C). Further western blotting experiments confirmed that LY294002 and Wortmannin inhibited the endogenous Akt phosphorylation (Fig. 4D). Overexpression of the miR-210 significantly promoted lipid accumulation in the presence of PI3K inhibitors, LY294002 and Wortmannin (Fig. 4E,F). Together with the western blot, these findings indicated that miR-210 might promote 3T3-L1 adipogenesis *via* activation on PI3K/Akt pathway.

MiR-210 POSITIVELY REGULATES PI3K/AKT PATHWAY BY TARGETING *SHIP1*

The SH2-containing inositol-5′-phosphatase (SHIP1), is a phosphatidylinositide phosphatase that negatively regulates PI3K-mediated signaling and hence represses the cell proliferation, differentiation, and survival. Deuter-Reinhard et al. [1997] demonstrated that forced expression of SHIP1 in *Xenopus* oocytes repressed germinal vesicle break down induced by insulin and hence inhibited oocyte maturation. Even though *Xenopus* oocytes did not express endogenous SHIP1, the effects of SHIP1 in response to insulin were directly correlated with decreased intracellular levels of PtdIns(3,4,5)P3

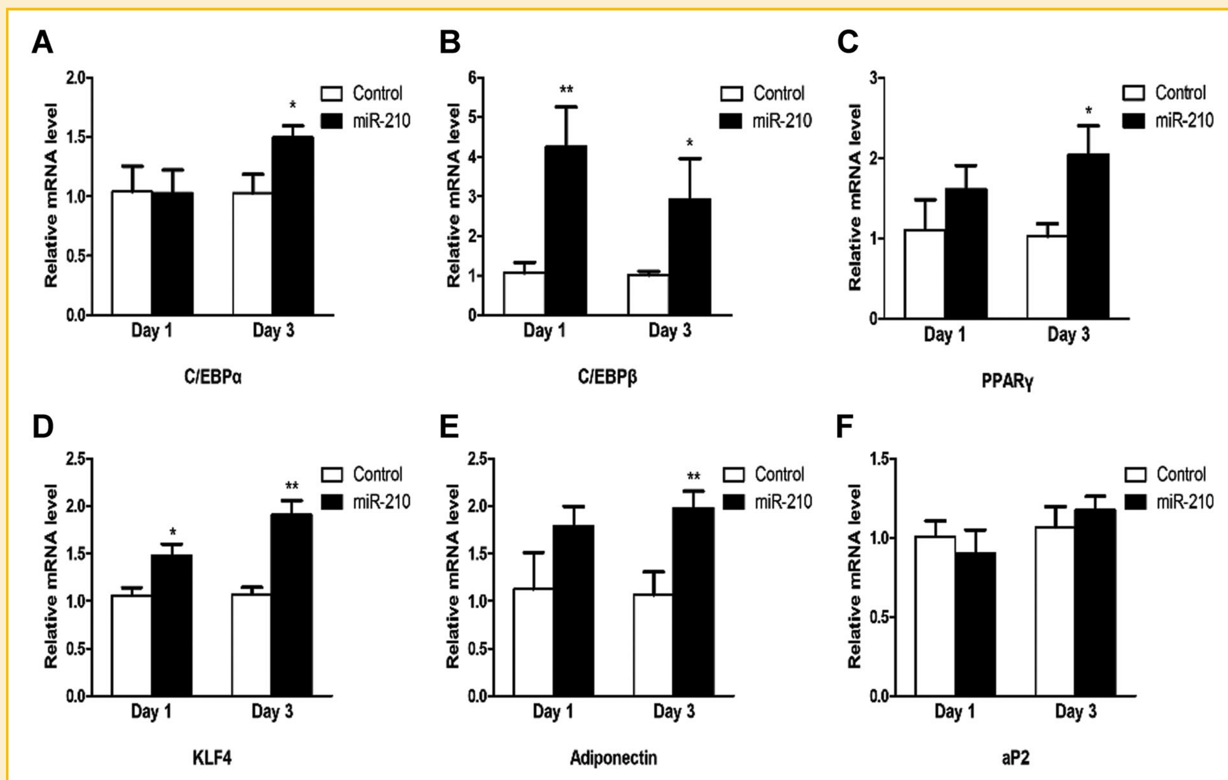

Fig. 2. Ectopic introduction of miR-210 increased the adipogenic marker expression. qRT-PCR analysis of the expression of the adipogenic marker genes C/EBPα (A), C/EBPβ (B), PPARγ (C), KLF4 (D), Adiponectin (E), and aP2 (F). The data shown are mean value ± standard errors of the mean from three independent experiments ($n = 3$; $^{*}P < 0.05$; $^{**}P < 0.01$).

[Deuter-Reinhard et al., 1997]. GLUT4 is an insulin-regulated glucose transporter found in adipose tissues that is responsible for insulin-regulated glucose uptake. Under low insulin concentration, GLUT4 is sequestered in intracellular vesicles whereas insulin could increase glucose absorption through relocating GLUT4 towards the plasma membrane. Vollenweider et al. [1999] showed that upregulation of SHIP1 by nuclear microinjection in 3T3-L1 adipocytes inhibited insulin-induced GLUT4 translocation while forced expression of the catalytically inactive mutant of SHIP1 abolished the insulin-induced relocation of GLUT4. Together, these results suggest a potential role of SHIP1 in regulating adipogenesis *via* negatively regulating PI3K/Akt pathway and relocating GLUT4. Lee et al. reported constitutively activated Akt and uniformly dramatic decreased SHIP-1 expression in acute myeloid leukemia (AML). Overexpression of SHIP-1 impaired myeloid colony growth, and SHIP-1 was a bona fide target of miR-210.

To determine whether miR-210 could modulate the expression of SHIP1 in mouse, mRNA level of SHIP1 were determined under the condition with or without hormonal induction. In agreement with the previous result in myeloid leukemia cell line [Lee et al., 2012], ectopic expression of miR-210 significantly reduced the mRNA level of SHIP1 without hormonal induction (Fig. 4G) or with hormonal induction (Fig. 4H).

In order to test whether SHIP1 might be regulated by miR-210, the luciferase reporter assay was conducted. HEK293 cells were cotransfected with miRNA mimics and a recombinant plasmid with the luciferase reporter gene fused with the 3′UTR region from mouse *SHIP-1* gene. Compared with negative control, overexpression of miR-210 decreased luciferase reporter activity by 35%, implying that miR-210 did interact with 3′UTR region of *SHIP-1* (Fig. 4I). To confirm the mutual interaction between miR-210 and 3′-UTR of *SHIP1*, we deleted the miR-210-targeting site in the 3′UTR of *SHIP1* and found that this deletion abolished the aforementioned inhibitory effect from miR-210 (Fig. 4I). Taken together, these results strongly indicated that miR-210 regulated 3T3-L1 differentiation in part by targeting *SHIP1*.

BLOCKADE OF miR-210 INHIBITS ADIPOGENESIS

To test the hypothesis that endogenous miR-210 is required for 3T3-L1 adipogenesis, we employed miR-210 inhibitor designed to inhibit endogenous miRNA molecules activity specifically during 3T3-L1 adipocyte differentiation. To increase the transfection efficiency, anti-miR-210 or negative control were transfected into 3T3-L1 cells three times every 2 days. Further qRT-PCR analysis verified a 0.5-fold repression on miR-210 expression level (data not shown). According to the Oil Red O staining, a significant repression on lipid storage was observed in anti-miR-210-transfected 3T3-L1 cells at day 6 post-confluence (Fig. 5A, B).

We also investigated the effect of anti-miR-210 on the expression of the well-characterized adipogenic marker genes such as PPARγ

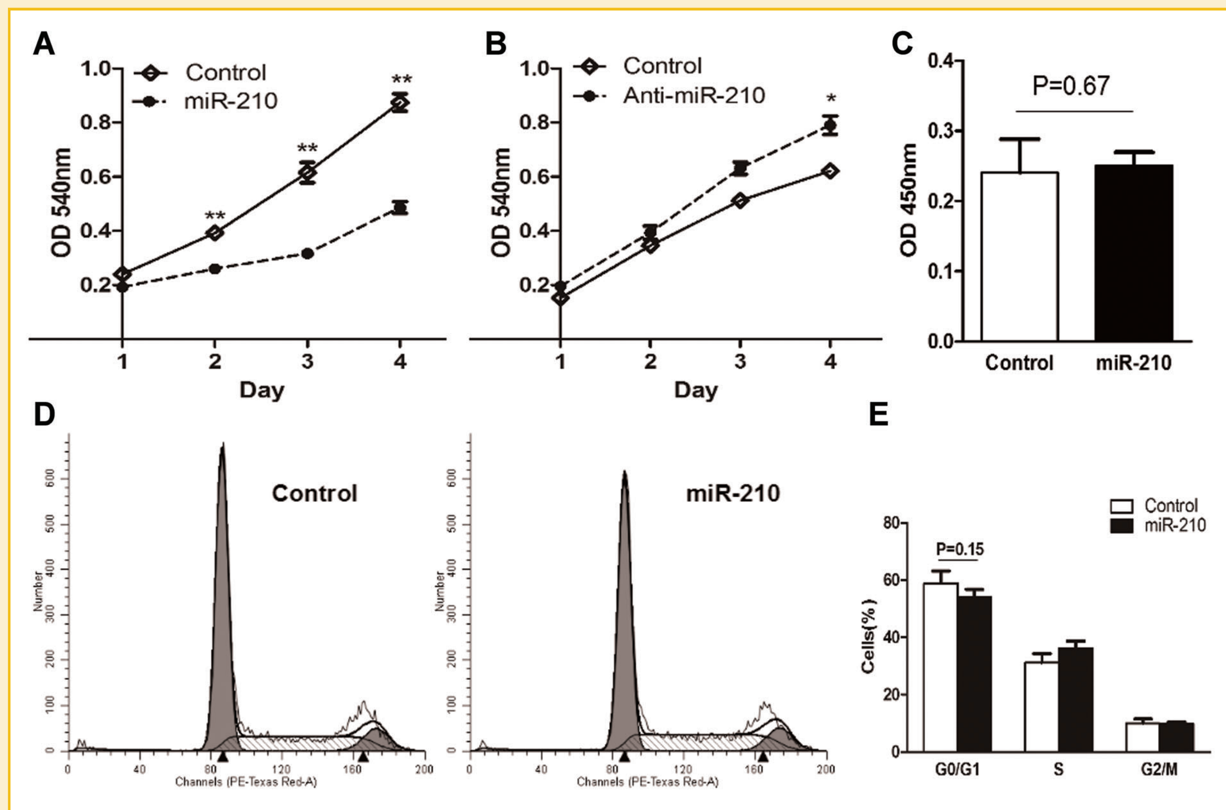

Fig. 3. Ectopic presence of miR-210 attenuated 3T3-L1 clonal expansion but not affect G1/S phase transition. The effect of miR-210 (A) or anti-miR-210 (B) on cell proliferation was determined by MTT assay (n = 3; $^{*}P < 0.05$; $^{**}P < 0.01$). C: The effect of miR-210 on DNA synthesis was determined by BrdU incorporation assay (n = 3; $^{*}P = 0.67$). D and E: The effect of miR-210 on G1/S phase transition was analyzed by cell flow cytometry (n = 3; $^{*}P = 0.15$).

and C/EBPα. Their respective mRNA levels were determined in 3T3-L1 cells on day 1 and day 3 during differentiation. On day 1, the expression level of most adipogenic marker genes were repressed by miR-210 inhibitor (Fig. 5D-G). On day 3, all adipogenic marker genes reduced >1.5-fold after transfection with miR-210 inhibitor.

Together, these results indicate a potential important role of endogenous miR-210 in regulating 3T3-L1 adipogenesis.

DISCUSSION

In this article, we identified miR-210 as a positive regulator of 3T3-L1 adipogenesis. First of all, we confirmed a dramatic increased expression of miR-210 during adipogenesis (Fig. 1B) [Sun et al., 2009; Qin et al., 2010]. Secondly, forced expression of miR-210 yielded increased lipid accumulation and upregulation of several adipogenic marker genes such as PPARγ and C/EBPα (Fig. 2). Finally, miR-210 activated PI3K/Akt pathway through repression on SHIP1 expression (Fig. 4). Altogether, these findings suggested that miR-210 emerged as a positive regulator of adipogenesis in the 3T3-L1 cell model.

It's well known that adipocytes and osteoblasts originated from the same mesenchymal progenitor cells, and bone marrow adipocytes possessed the capacity to differentiate into osteoblasts in some cases [Justesen et al., 2004]. 3T3-L1 cells, an immortalized preadipocyte cell line that was established from subclones of 3T3 mouse embryonic fibroblasts, also appeared to have the ability to differentiate into osteoblasts [Zhang et al., 2006; Takahashi, 2011]. In spite of intensive research efforts focusing on the individual differentiation pathways, however, the molecular mechanism underlying the conversion from 3T3-L1 preadipocyte into osteoblasts remains elusive. Currently, growing evidence indicates that miRNA emerges as an important player in regulating gene expression in diverse cellular processes. More and more studies indicate that miRNAs act as vital mediators to fine-tune the balance between adipogenesis and osteogenesis [Huang et al., 2010a,b, 2012; Zhang et al., 2011]. For instance, Mizuno et al. [2009] reported that miR-210 was upregulated during osteoblastic differentiation and overexpression of miR-210 positively regulated osteoblastic differentiation of mouse mesenchymal ST2 cells through targeting the activin A receptor type 1B (AcvR1b) gene. Thus, together with our study in adipogenesis, the analogous function of miR-210 was found in both osteoclastogenesis and adipogenesis. However, previous studies suggest that the transdifferentiation of mesenchymal stem cells (MSCs) into adipocytes or osteoblasts is competitively balanced [Muruganandan et al., 2009], indicating an elusive role of miR-210 in regulating the balance between osteoclastogenesis and adipogenesis.

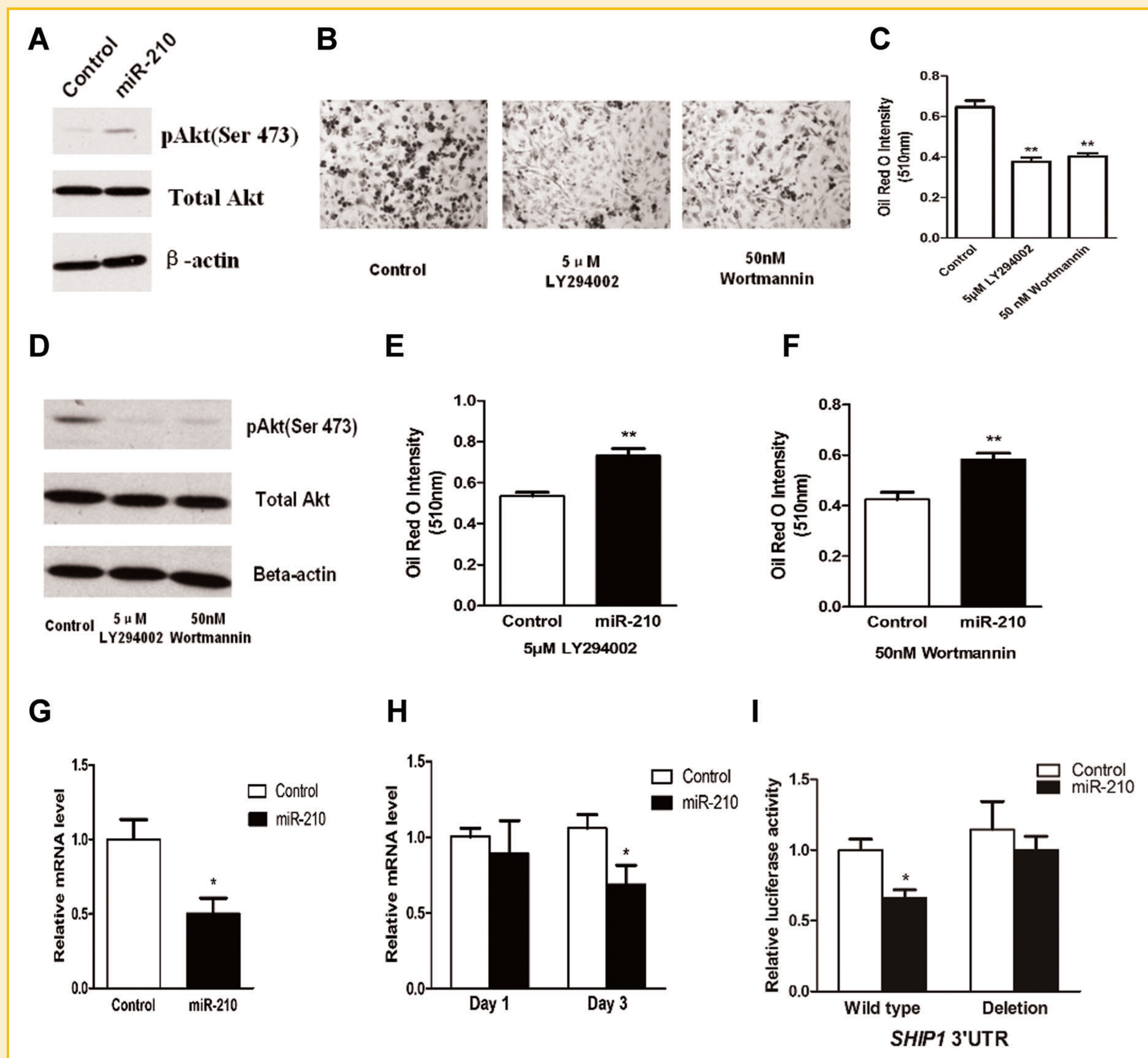

Fig. 4. MiR-210 promoted 3T3-L1 adipogenesis through activation of PI3K pathway by targeting SHIP1. A: Representative western blot results showed phosphorylated Akt (Ser473) and total Akt level after transfection with miR-210. B: Representative microscopic images of lipid accumulation were taken during 3T3-L1 adipogenesis after the treatment of LY294002 and Wortmannin (magnification, 200×). C: The effect of LY294002 and Wortmannin in suppressing the 3T3-L1 adipogenesis was quantified after extraction with isopropanol by reading at 510 nm (n = 3; $^{**}P < 0.01$). D: Representative western blot results showed phosphorylated Akt (Ser473) and total Akt level after the treatment of LY294002 and Wortmannin. E and F: The effect of miR-210 in rescuing the inhibition of adipogenesis by the treatment of PI3K inhibitor LY294002 (E) and Wortmannin (F) was determined by Oil Red O staining (magnification, 200×). The amount of Oil Red O was quantified after extraction with isopropanol by reading at 510 nm (n = 3; $^{**}P < 0.01$). G and H: The mRNA level of SHIP1 after transfection with miR-210 with (G) or without (H) hormonal induction was analyzed by qRT-PCR (n = 3; $^{*}P < 0.05$). I: pMIR-REPORT Beta-gal vector (internal control), pMIR-REPORTSHIP1 3′UTR-wt, or pMIR-REPORT-SHIP1 3′UTR-deletion luciferase vector were cotransfected with miR-210 mimics or negative control. Firefly luciferase activity was normalized to β-galactosidase activity. The luciferase activity of miR-210-transfected group was normalized to that of negative control-transfected group (n = 3; $^{*}P < 0.05$).

Interestingly, emerging evidences confirmed that miR-210 was the most consistently and predominantly upregulated miRNA under hypoxia in diverse systems [Huang et al., 2010a,b]. In view of the fact that hypoxia promoted osteoblastic differentiation [Hirao et al., 2007] but repressed adipocyte differentiation [Yun et al., 2002], it raises the possibility that hypoxia contributes to osteoclastogenesis partially *via* activation of miR-210 expression, whereas miR-210 may be merely a passenger in the process of adipogenesis due to incidental regulation by other unknown transcription factors. However, another acceptable explanation for this controversial issue is that miR-210 might be essential for both osteoclastogenesis and adipogenesis but it functions in different stages of differentiation and hence its upregulation could be detected in these two opposing biological processes. Nevertheless, further investigations are needed to shed light on the molecular mechanism underlying the role of miR-210 in regulating the balance between osteoclastogenesis and adipogenesis.

The PI3K/Akt signaling has been shown to play a unique role in lipid accumulation and glucose homeostasis, based on the dramatic phenotype changes in knockout mice as well as diverse different

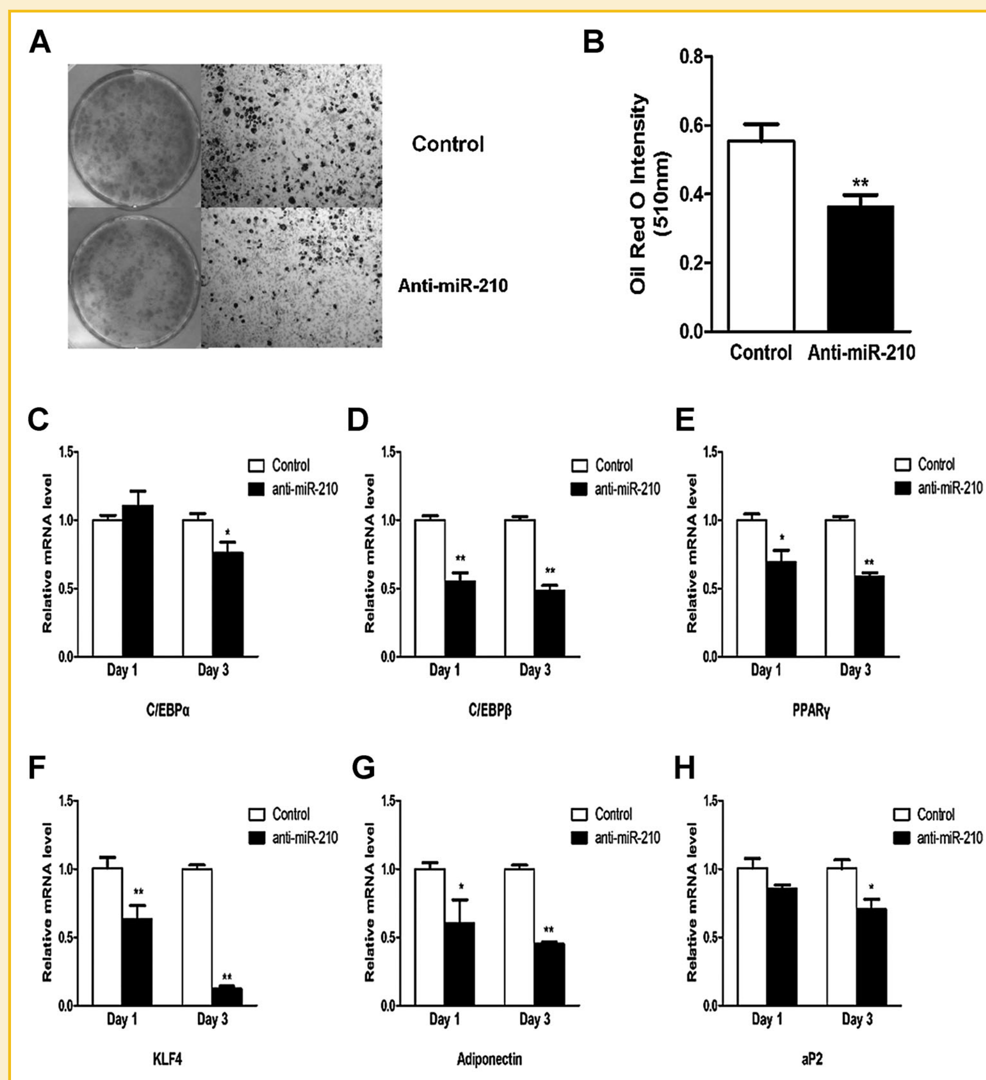

Fig. 5. Blockade of endogenous miR-210 level inhibited adipogenesis. A: The effect of anti-miR-210 in 3T3-L1 adipocyte differentiation was determined by Oil Red O staining (magnification, 200×). B: The amount of Oil Red O was quantified after extraction with isopropanol by reading at 510 nm (n = 3; $^{**}P < 0.01$). C–G: qRT-PCR analysis of the expression of the adipogenic marker genes C/EBPα (C), C/EBPβ (D), PPARγ (E), KLF4 (F), Adiponectin (G), and aP2 (H) after transfection with anti-miR-210 (n = 3; $^{*}P < 0.05$; $^{**}P < 0.01$).

preadipocyte cell lines. Akt1/Akt2 double-knockout mice exhibited an impaired capacity to differentiate into mature adipocytes [Peng et al., 2003; Baudry et al., 2006; Yun et al., 2008]. The constitutively active Akt relocated the Glucose transporter type 4 (GLUT4) to the plasma membrane and increased glucose uptake and hence provoke 3T3-L1 adipogenesis [Vollenweider et al., 1999]. Forced expression of SHIP1 *via* nuclear microinjection in 3T3-L1 cells halted insulin-induced GLUT4 translocation towards plasma membrane [Vollenweider et al., 1999]. One major effect of insulin was to facilitate GLUT4 translocation and glucose uptake in insulin-sensitive tissues, and it has been well documented that PI3K activation was both necessary and sufficient for these actions [Haruta et al., 1995; Martin et al., 1996; Zhang et al., 2009]. Hence, our data showed that miR-210-mediated inhibition in SHIP1 expression may promote insulin-induced GLUT4 translocation and hence increase glucose uptake for adipogenesis.

To sum up, we provided evidence that miR-210 positively regulated adipocyte differentiation and proposed that miR-210 did this partially by targeting SHIP1, therefore activated Akt phosphorylation. Our findings also suggest that miR-210 may potentially play a role in obesity-related metabolic diseases.

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