Regulation of adipogenic differentiation by LAR tyrosine phosphatase in human mesenchymal stem cells and 3T3-L1 preadipocytes

Won-Kon Kim1,2, Hyeyun Jung1, Do-Hyung Kim1, Eun-Young Kim1, Jin-Woong Chung3, Yee-Sook Cho4, Sung-Goo Park1, Byoung-Chul Park1, Yong Ko2, Kwang-Hee Bae1,* and Sang-Chul Lee1,*

1Medical Proteomics Research Center, and 2Development and Differentiation Research Center, KRIBB, Daejeon 305-806, Republic of Korea
3Division of Life Science and Genetic Engineering, College of Life and Environmental Sciences, Korea University, Seoul 136-701, Republic of Korea
4Department of Biological Sciences, Dong-A University, Busan 604-714, Republic of Korea

*Authors for correspondence (khbae@kribb.re.kr; lesach@kribb.re.kr)

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Summary
Mesenchymal stem cells (MSCs) are multipotent adult stem cells that can differentiate into a variety of mesodermal-lineage cells. MSCs have significant potential in tissue engineering and therapeutic applications; however, the low differentiation and proliferation efficiencies of these cells in the laboratory are fundamental obstacles to their therapeutic use, mainly owing to the lack of information on the detailed signal-transduction mechanisms of differentiation into distinct lineages. With the aid of protein-tyrosine-phosphatase profiling studies, we show that the expression of leukocyte common antigen related (LAR) tyrosine phosphatase is significantly decreased during the early adipogenic stages of MSCs. Knockdown of endogenous LAR induced a dramatic increase in adipogenic differentiation, whereas its overexpression led to decreased adipogenic differentiation in both 3T3-L1 preadipocytes and MSCs. LAR reduces tyrosine phosphorylation of the insulin receptor, in turn leading to decreased phosphorylation of the adaptor protein IRS-1 and its downstream molecule Akt (also known as PKB). We propose that LAR functions as a negative regulator of adipogenesis. Furthermore, our data support the possibility that LAR controls the balance between osteoblast and adipocyte differentiation. Overall, our findings contribute to the clarification of the mechanisms underlying LAR activity in the differentiation of MSCs and suggest that LAR is a candidate target protein for the control of stem-cell differentiation.

Key words: Adipocyte, Adipogenesis, Mesenchymal stem cells, Osteoblast, Protein tyrosine phosphatase, Leukocyte common antigen related

Introduction
Adipocytes are highly specialized cells that have an important role in energy homeostasis by harboring energy reservoirs as lipid droplets (Cornelius et al., 1994; Hwang et al., 1997). However, these reservoirs are implicated in a host of major human health problems, because excessive or insufficient energy reserves result in metabolic disorders, such as obesity and lipodystrophy (Garg, 2000; Otto and Lane, 2005). Adipogenesis involves the formation of preadipocytes from mesenchymal progenitor cells and their subsequent differentiation into adipocytes (Greigore et al., 1998; Rosen and Spiegelman, 2000). The cellular and molecular mechanisms of adipocyte differentiation are regulated via activation of several adipogenic intracellular signaling pathways.

Insulin signaling clearly has marked effects on adipogenesis. The mechanisms of insulin are mediated by a cascade of tyrosine-phosphorylation events initiated by the binding of insulin to its receptor (White and Kahn, 1994; White, 1997; White and Yenush, 1998). Binding stimulates the kinase activity of the insulin receptor (IR) and the phosphorylation of IR substrates (IRSs), leading to the activation of downstream signaling molecules, including phosphoinositide 3-kinase (PI3K) and Akt (also known as PKB). Activated Akt regulates the activity of several downstream proteins involved in gluconeogenesis, lipogenesis and adipogenesis (White and Kahn, 1994; Wang and Sul, 1998; Czech and Corvera, 1999; Withers et al., 1999; Baudry et al., 2006; Rosen and MacDougald, 2006). Downstream components of the insulin signaling cascade are crucial for adipogenesis. The loss of individual IRS proteins, including the combined deletion of IRS1 and IRS2, leads to inhibition of adipogenesis (Laustsen et al., 2002; Tseng et al., 2004; Rosen and MacDougald, 2006). Moreover, inhibition of PI3K and the loss of Akt repress adipogenesis via regulation of adipogenic and anti-adipogenic transcription factors (Garofalo et al., 2003; Nakae et al., 2003; Wofrum et al., 2003; George et al., 2004; Menghini et al., 2005; Rosen and MacDougald, 2006).

Numerous growth-factor and -hormone receptors belong to the tyrosine-kinase-receptor family and undergo phosphorylation and dephosphorylation at tyrosine residues in a concerted manner in response to a stimulus in order to initiate the signaling cascade (Meng et al., 2004; Niu et al., 2007). Termination of insulin activity is also mediated by protein tyrosine phosphatases (PTPs), which dephosphorylate and inactivate the IR and, subsequently, post-receptor substrates. PTPs constitute a large family of transmembrane or intracellular enzymes that function as either positive or negative regulators of a number of signaling pathways (Goldstein, 1993; Denu et al., 1996; Tonks and Neel, 1996). Several PTPs, such as leukocyte common antigen related (LAR; also known as PTP-RF), phophatase, PTP-α, PTP-1B and SHPTP2 (also known as Syp), are highly expressed in the major insulin-sensitive tissues, including the liver, skeletal muscle and adipose tissue. These enzymes are involved in insulin signaling, as established using a variety of
LAR is a receptor-type PTP that has two tandem-repeat cytoplasmic phosphatase domains (D1 and D2), of which the membrane-proximal D1 domain possesses catalytic activity (Strueli et al., 1990; Nam et al., 1999). LAR is widely expressed in insulin-sensitive tissues. Increased association of LAR with the IR occurs after treatment with insulin (Ahmad et al., 1995; Ahmad and Goldstein, 1997). Several studies have reported that LAR is negatively regulated during insulin signaling. Moreover, overexpression of LAR leads to suppression of insulin activity, and knockdown of LAR enhances IR phosphorylation and PI3K activity in hepatoma cells (Kulas et al., 1995; Kulas et al., 1996; Li et al., 1996). An in vivo study similarly reported that overexpression of LAR in mouse skeletal muscle suppresses IR signaling (Zabolotny et al., 2001). LAR also regulates insulin-like growth factor-1 receptor signaling in vascular smooth-muscle cells (Niu et al., 2007). These reports strongly implicate LAR in insulin-mediated adipocyte differentiation. There are no direct reports on the exact role of LAR in adipogenesis, although earlier studies have demonstrated that LAR expression is decreased during adipogenesis of human mesenchymal stem cells (MSCs) (Song et al., 2006). The present study was undertaken to determine the regulatory effects of LAR on the differentiation of preadipocytes and human MSCs into adipocytes.

**Results**

**Expression of LAR during the differentiation of MSCs and 3T3-L1 cells into adipocytes**

To determine the roles of the PTP family during the early phase of adipogenesis, we extensively assessed the expression changes of all PTP members via reverse-transcriptase PCR (RT-PCR) analysis (data not shown). Interestingly, expression of LAR mRNA was markedly decreased during adipocyte differentiation of human MSCs (Fig. 1A). To obtain a detailed assessment of the LAR level, real-time PCR analysis was performed. LAR mRNA expression was clearly decreased at the later as well as the early stages of adipogenesis in MSCs (Fig. 1B). Next, we assessed LAR expression in 3T3-L1 preadipocyte cell lines using both RT-PCR and western blot analyses. As shown in Fig. 1C,D, LAR expression levels decreased during the differentiation of 3T3-L1 preadipocytes. For RT-PCR analysis, total RNA was extracted on the indicated days of differentiation and used to synthesize CDNA for RT-PCR. Two primer sets were used to check for LAR expression, one for amplification of the mRNA region encoding the extracellular domain (Extra LAR) and another for amplification of the mRNA region encoding the cytoplasmic domain (Cyto LAR). The results obtained using the two sets of primers revealed similarly decreased LAR expression patterns during adipogenesis of 3T3-L1 cells. αP2 and PPAR-γ were used as positive controls for adipocyte differentiation and β-actin was used as a loading control. For western blot analysis, whole-cell lysates were extracted on the indicated days of differentiation, and subjected to analysis using antibodies against LAR and adipocyte-differentiation markers, such as αP2 and PPAR-γ. β-actin was used as a loading control.

**Depletion of LAR promotes the differentiation of 3T3-L1 preadipocytes into mature adipocytes**

To determine whether endogenous LAR influences differentiation into adipocytes, we infected 3T3-L1 preadipocytes with a retrovirus expressing LAR shRNA, scrambled insert or control vector only. Infected cells were isolated by FACS sorting and then cultured further. As shown in Fig. 2A (upper panels), cells infected with a retrovirus expressing shRNA against LAR were successfully enriched. Knockdown of endogenous LAR expression was confirmed by RT-PCR and western blot analyses (Fig. 2B). Next, retrovirally transduced 3T3-L1 preadipocytes were induced to differentiate into adipocytes for 2 days in growth medium containing MDI (see Materials and Methods), followed by further differentiation and maturation in growth medium supplemented with insulin for 6 days. In parallel, fat accumulation was visualized by staining lipid droplets with Oil red-O (Fig. 2C). Interestingly, depletion of LAR dramatically facilitated the differentiation of these cells into mature adipocytes compared with control and scrambled retrovirus-infected cells (Fig. 2D). Next, to exclude off-target effects of shRNA treatment, we attempted to rescue the effect on differentiation by re-introducing an shRNA-resistant cytoLAR or mutant LAR (D1-CS, activity-dead mutant) into the LAR-knockdown 3T3-L1 cells. Most of the re-infected cells were detected as both GFP- and RFP-positive by fluorescence microscopy (Fig. 2A, lower panels). Knockdown of endogenous LAR and expression of shRNA-resistant cytoLAR or mutant LAR D1-CS were confirmed by RT-PCR analysis (Fig. 2E).

Consistently, the knockdown of endogenous LAR mRNA levels was continuously maintained until 6 days after the adipogenic differentiation of 3T3-L1 cells (Fig. 2E). The re-introduction of wild-type cytoLAR into LAR-knockdown cells induced a full recovery of the differentiation rate. By contrast, the re-introduction of LAR D1-CS
Effects of LAR on adipocyte differentiation of human MSCs

LAR and specific shRNA against LAR were stably expressed in human MSCs via retroviral infection to establish the importance of LAR during the adipogenesis of human MSCs (Fig. 4A). LAR showed no significant changes in the differentiation rate of the cells, indicating the crucial involvement of LAR phosphatase activity in adipogenesis (Fig. 2F,G).

Ectopic expression of LAR inhibits the differentiation of 3T3-L1 preadipocytes into adipocytes

Depletion of LAR induced a dramatic increase in the adipocyte differentiation of 3T3-L1 cells, indicating that LAR phosphatase is involved in the negative regulation of adipocyte differentiation.
expression and knockdown were confirmed by western blot and RT-PCR analyses, respectively (Fig. 4B). Analogous to the data obtained with 3T3-L1 preadipocytes, knockdown of LAR promoted adipocyte differentiation of human MSCs (Fig. 4C,D), whereas LAR overexpression suppressed adipogenic differentiation (Fig. 4E,F).

LAR affects insulin-mediated adipogenic signal transduction
To clarify the mechanism underlying LAR-induced regulation of adipogenesis, we examined the phosphorylation states of IR, IRS-1, and its downstream target molecule Akt following treatment with insulin or MDI. Consistent with a previous report (Li et al., 1996), tyrosine phosphorylation of IR in 3T3-L1 preadipocytes was rapidly increased in response to insulin or MDI. Notably, overexpression of LAR led to the inhibition of insulin- and MDI-induced tyrosine phosphorylation of IR and IRS-1 (Fig. 5A,B). Consistently, this decrease in tyrosine phosphorylation resulted in reduced phospho-Akt levels in cells overexpressing LAR, compared with control cells (Fig. 5A,B). A marginal effect of activity-dead mutant LAR on the phosphorylation of IR, IRS-1 and Akt was detected, indicating that the phosphatase activity of LAR is directly involved in the regulation of adipogenesis. Next, we checked the phosphorylation states of IR, IRS-1 and its downstream target molecule Akt after knockdown of LAR using an shRNA construct. LAR knockdown induced an increase in the phosphorylation of IR and IRS-1, resulting in augmented phospho-Akt levels (Fig. 5C,D). These results strongly suggest that LAR functions as a negative regulator of adipogenesis by controlling the phosphorylation level of IR, IRS-1 and, consequently, Akt.

Effects of LAR on osteoblast differentiation
To establish whether LAR is involved in regulating osteoblastogenesis, we first examined the change in the expression level of LAR during osteoblastogenesis of MC3T3-E1 cells. As expected, the mRNA level of LAR was continuously increased during the differentiation of MC3T3-E1 cells into osteoblasts (Fig. 6A). LAR shRNA or cytoplasmic LAR was stably introduced into MC3T3-E1 preosteoblast cells using a retroviral system. Knockdown and overexpression of LAR were confirmed by fluorescence microscopy, western blot and RT-PCR analyses (Fig. 6B,C). Cells were differentiated into osteoblasts by culturing them in osteogenic induction medium for 21 days. Cells were then stained with Alizarin-Red-S solution to visually detect mineralization. Interestingly, cells depleted of LAR displayed a significantly lower degree of mineralization than control cells (Fig. 6D,E). By contrast, the degree of mineralization was significantly increased in cells overexpressing LAR, compared with control cells. LAR D1-CS

Fig. 4. Effects of LAR on adipogenic differentiation of human MSCs. MSCs were infected as described in Figs 2,3. (A) The infected cells were enriched by FACS sorting and confirmed by fluorescence microscopy. (B) The western blot (left) and RT-PCR (right) analyses were performed to confirm the overexpression and knockdown of LAR, respectively. (C) LAR-depleted human MSCs (using the shLAR-II construct) were induced into the adipogenic lineage for 14 days, according to standard procedures, and the cells were then stained with Oil red-O to visualize the degree of lipid accumulation. (D) Quantification of Oil red-O staining. Data represent the mean percentage levels ± s.d. compared with control vector (n=3; *P<0.05). (E) Human MSCs overexpressing the cytoLAR or LAR D1-CS mutant were induced to differentiate into adipocytes via the adipogenic program for 14 days. Cells were stained with Oil red-O to visualize lipid droplets at 14 days post-induction. (F) Quantification of stained cells was performed using dye extraction buffer. Data represent the mean percentage levels ± s.d. compared with control vector (n=3; *P<0.05).

Fig. 5. LAR dephosphorylates IR, IRS-1 and its downstream target Akt. (A,B) After obtaining confluent cultures of 3T3-L1 cells overexpressing the cytoLAR or LAR D1-CS mutant, cells were serum-starved for 12 hours and treated with 100 ng/ml insulin (A) or MDI (B) for the indicated times. (C,D) In the case of LAR-knockdown 3T3-L1 cells (by shLAR-II), cells were cultured to confluence, serum-starved for 12 hours and treated with 100 ng/ml insulin (C) or MDI (D) for the indicated times. (A-D) Total cell lysates containing equal amounts of protein were immunoprecipitated with anti-IR-β and anti-IRS-1 antibodies, and western blot analysis was performed with anti-phosphotyrosine (4G10; anti-pY). The same membrane was reprobed and immunoblotted with anti-IR-β and IRS-1 antibodies. Akt phosphorylation was measured with phospho-Akt antibody (antibody to phospho-Ser473) and the same membrane was immunoblotted with anti-Akt antibody.
Tyrosine phosphorylation is one of the fundamental mechanisms underlying the numerous important aspects of eukaryote physiology, including cell growth and differentiation. Adipocyte differentiation is regulated by tyrosine phosphorylation balanced by the antagonistic actions of protein tyrosine kinases (PTKs) and PTPs (Hunter, 1987; Hunter, 1998; Blume-Jensen and Hunter, 2001).

Discussion

Tyrosine phosphorylation is one of the fundamental mechanisms underlying the numerous important aspects of eukaryote physiology, including cell growth and differentiation. Adipocyte differentiation is regulated by tyrosine phosphorylation balanced by the antagonistic actions of protein tyrosine kinases (PTKs) and PTPs (Hunter, 1987; Hunter, 1998; Blume-Jensen and Hunter, 2001).
et al., 2004). Interestingly, in contrast to adipogenic differentiation, osteoblast differentiation was augmented upon LAR overexpression and reduced following LAR depletion. This reciprocal effect of LAR on adipogenesis and osteoblastogenesis is consistent with previous findings that GILZ (glucocorticoid-induced leucine zipper) induces osteoblastogenesis and inhibits adipogenesis in MSCs, and that TAZ (transcriptional co-activator with PDZ-binding motif) controls the balance of osteoblast and adipocyte differentiation (Hong et al., 2005; Zhang et al., 2008).

In conjunction with earlier data, our results suggest that inhibition of adipogenic differentiation by LAR occurs through the enhancement of osteogenic differentiation. Thus, LAR might act as a modulator of the balance between osteoblast and adipocyte differentiation in MSCs. However, the reason for the marginal effect of LAR on osteoblast differentiation, compared with that on adipocyte differentiation, remains to be defined. Thus, extensive studies to establish the mechanism underlying LAR involvement in osteoblastogenesis are warranted. In addition, LAR knockout (KO) mice were smaller in size than wild-type mice, were insulin-resistant and exhibited subtle alterations in neuroscience, although they have been reported to be, in general, normal and to possess no adipose-tissue or bone morphogenesis defects (Ren et al., 1998). Therefore, to elucidate the detailed functions of LAR in adipogenesis and osteoblastogenesis at an organism level, it is necessary to conduct more detailed investigations of LAR KO mice.

Materials and Methods

Cell culture

The preadipocyte cell line 3T3-L1, derived from mouse embryo fibroblasts, was cultured in growth medium [high glucose Dulbecco’s modified Eagle’s medium (DMEM) containing 1% antibiotic-antimycotic solution and 10% bovine calf serum (BCS); Gibco-Invitrogen] at 37°C in a humidified atmosphere with 5% CO₂. MC3T3-E1 preosteoblasts were purchased from ATCC and cultured in maintenance medium (α-mineral essential medium (MEM) with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution). Human MSCs were purchased from Cambrex Bio Science and maintained in MSC growth medium (MSCGM; Cambrex Bio Science) at 37°C in a humidified atmosphere with 5% CO₂.

Differentiation into adipocytes

3T3-L1 cells were induced to differentiate into mature adipocytes, as described previously (Kim et al., 2008; Jung et al., 2009). Briefly, at 2 days after confluence (day 0), cells were placed in differentiation medium composed of DMEM, 10% FBS and 10 μM dexamethasone and 10 μM isobutylmethylxanthine (IBMX); Sigma). To induce osteoblastogenesis, confluent MC3T3-E1 cells were switched to osteogenic induction medium (50 μM β-glycerophosphate, 1 μM dexamethasone and 100 μM ascorbic acid; Sigma) and the medium was changed every 2-3 days. The mixture was incubated overnight at 4°C. Protein A-G plus agarose beads (Calbiochem) were added, followed by agitation for 1 hour at 4°C. Immunoprecipitates were recovered by centrifugation at 2500 × g. washed five times with NP-40 lysis buffer and resuspended in sodium dodecyl sulfate (SDS) sample buffer.

Differentiation into osteoblasts

To induce osteoblastogenesis, confluent MC3T3-E1 cells were switched to osteogenic induction medium (α-MEM with 10% FBS) supplemented with 50 μM β-glycerophosphate and 10 mM dexamethasone; Sigma) and the medium was changed every 2-3 days.

Construction of retroviral vectors and transduction

To construct 3T3-L1 cells and MSCs stably expressing the FLAG-tagged cytoplasmic domain of human LAR (spans 1316-1897), a retrovirus-mediated infection system was used. For expression of cytoplasmic LAR, DNA encoding FLAG-tagged cytoplasmic LAR was inserted into the multi-cloning site of the pRetroX-IRES-ZsGreen1 vector (Clontech). Retroviruses were subsequently produced by transfecting co-transfected GP-293 cells with a retroviral vector and VSV-G plasmid using Lipofectamine 2000 (Gibco-Invitrogen). At 48 hours after transfection, media containing retroviruses were collected, filtered with 0.45-μm filters and used to infect cells in the presence of polybrene (8 μg/ml). Infected cells were selected using a FACS/Artia cell sorter (BD Bioscience) and further maintained in growth medium. qPCR expression of FLAG-tagged cytoplasmic LAR was confirmed by western blot analysis. As a negative control, a cytoplasmic LAR mutant (LAR D1-CS; inactive mutant; catalytic Cys1522 replaced with Ser) was constructed (Streuli et al., 1990; Nam et al., 1999).

RNA interference

To knock down LAR in 3T3-L1 cells, MC3T3-E1 cells and MSCs, the pSIREN-RetroQ-DsRed Express retroviral vector (Clontech) was employed. shRNAs were designed by selecting a target sequence specific for mouse and human LAR genes, as described previously (Mander et al., 2005; Bernabeu et al., 2006). The following gene-specific sequences were used to successfully inhibit LAR expression in both humans and mice: shLAR-I top: 5'-GATGCGGAATTCACTGGTGAGTAAAATCCAAAAGAAGATTCTTCATCCACGAGTCATTCTCTACACGTTTTCGTTTTTGG-3', bottom: 5'-AATTCAAAAAAAGAATACGCGGATGGAAGAATCTCTTGAATCTTTCATCCACGAGTCATTCTCTACACGTTTTCGTTTTTGG-3', bottom: 5'-AATTC-AAAAAGAATACGCTTATACCTACAGCTCTGGTAGACATGCTTATGCTTTTG-3'. The constructs possess an altered mRNA sequence in the region corresponding to the shRNA sequence. The constructs were obtained by standard methods using the primers 5'-GGCCTAATAGCGACACAGGGGCCTC-3' and 5'-TTCCTGCTTTCAATACCAATCCAGGCA0A-3'. Underlined regions indicate the mutated sites (mutations did not induce a change in amino acid residues).

Immunoblot analysis

Cells were washed three times with ice-cold phosphate buffered saline (PBS) containing 1 mM sodium orthovandate and harvested in ice-cold RIPA or NP-40 lysis buffer containing an inhibitor cocktail (Roche). Protein concentrations were measured with the BCA protein assay kit (Pierce). SDS-PAGE, western blot and densitometric analyses were performed using standard protocols. The anti-LAR antibody is described in a previous report (Niu et al., 2007). Anti-α-p2, anti-α-PAR-γ and anti-phospho-Akt (cat. no. #9271S; antibody to phosphorylated T-loop of the Akt ser/thr family) were purchased from Invitrogen, whereas anti-FLAG and anti-β-actin antibodies were from Sigma. The secondary antibodies were purchased from Abcam, and membranes were visualized using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce).

Immunoprecipitation

For immunoprecipitation, anti-IR or anti-IR-1 antibodies (1:50-1:100 dilution) were added to lysates containing equal amounts of protein (200-300 μg). The mixture was incubated overnight at 4°C. Protein A-G plus agarose beads (Calbiochem) were added, followed by agitation for 1 hour at 4°C. Immunoprecipitates were recovered by centrifugation at 2500 × g, washed five times with NP-40 lysis buffer and analyzed as described above.

RNA extraction and real-time PCR

Total RNA was extracted from cultured cells using RNeasy mini columns (QIAGEN), and first-strand complementary DNA (cDNA) was synthesized using 1 μg of total RNA as template, 500 μg of oligo (dT) and AccuPower RT Premix (Bioneer, Korea) in a total volume of 20 μl, according to previously described methods (Jung et al., 2009). The primer sequences were as follows: mouse osteopontin LAR (forward, 5'-GTCAGAAGCTCTGTTAACACCACT-3'), reverse, 5'-CCGCTTCCTTGGTCTTTCA-3'; mouse extracellular region of LAR (forward, 5'-CAACGATCCACGGAAGTGTCT-3'), reverse, 5'-GGGGCAGAGGATTTGCTG3'-3'; mouse LAR (forward, 5'-ACCGATGCTCTGAGTACAC-3'), reverse, 5'-GGCCTGCTTTCAATACCAATCCAGGCA0A-3'. The targeted cDNA fragment of each differentiation-associated gene was amplified by PCR using 5 μl of the reverse transcription product, 10 μl of each primer pair and the AccuPower PCR premix (Bioneer, Korea). PCR products were separated by electrophoresis in 2% agarose gels and visualized by staining with ethidium bromide.

For the real-time PCR, SYBR Premix Ex Taq (TakaRa) was applied to detect the LAR expression level using a Dice TP 800 Thermal Cycler (TakaRa). The following primers were used for human LAR (forward, 5'-CCCCTCTAAACCAAGTGGGCAAG-3', reverse, 5'-CCCTCGAAGGAGCTGTTATAAAG-3') and β-actin.
Oil-red-O staining

Lipid droplets in differentiating or mature adipocytes were stained using the Oil-red-O method, as described previously (Kim et al., 2008; Jung et al., 2009). In brief, cultured cells were washed twice with PBS, fixed for 30 minutes with 10% formalin and washed twice with distilled water prior to staining. Lipid droplets within the cells were stained for 30 minutes using 0.3% filtered Oil-red-O solution in 60% isopropanol (Sigma). The cells were then washed twice with distilled water and micrographs were obtained. Oil-red-O staining was then quantified, as described previously (Ramírez-Zacarías et al., 2009). After elution from fixed cells with absolute isopropanol, the extracted dye was measured with a GeneQuant 1300 spectrophotometer (GE Healthcare) at 510 nm.

Alizarin-Red-S staining

Alizarin-Red-S staining and mineral-content quantitation were performed following earlier procedures with minor modifications (Stanford et al., 1995). Briefly, the cells were washed with calcium and phosphate-free saline solution, and fixed with 10% formalin for 30 minutes. After washing with distilled water, the mineral content was stained with 40 mM Alizarin-Red-S solution (pH 4.2, Sigma) at room temperature for 30 minutes. After washing with distilled water, the mineral content was eluted from fixed cells with absolute isopropanol, the extracted dye was measured with a GeneQuant 1300 spectrophotometer (GE Healthcare) at 510 nm.

Statistical analysis

All quantitative data were analyzed using an independent Student’s t-test and considered as significant at P<0.05.

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