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Control of Adipogenesis by the Autocrine Interplays between Angiotensin 1–7/Mas Receptor and Angiotensin II/AT_1 Receptor Signaling Pathways*[S]

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Background: The autocrine regulation of Ang(1–7) upon adipogenesis is unknown.
Results: The autocrine counteractive interplays between Ang(1–7)-Mas and AngII-AT_1 signaling upon adipogenesis are revealed.
Conclusion: The Ang(1–7)-Mas activation stimulates adipogenesis and antagonizes the antiadipogenic effect of AngII-AT_1 activation.
Significance: The angiotensin system in adipose tissue may serve as a potential therapeutic target for obesity and related metabolic disorders.

Angiotensin II (AngII), a peptide hormone released by adipocytes, can be catabolized by adipose angiotensin-converting enzyme 2 (ACE2) to form Ang(1–7). Co-expression of AngII receptors (AT_1 and AT_2) and Ang(1–7) receptors (Mas) in adipocytes implies the autocrine regulation of the local angiotensin system upon adipocyte functions, through yet unknown interactive mechanisms. In the present study, we reveal the adipogenic effects of Ang(1–7) through activation of Mas receptor and its subtle interplays with the antiadipogenic AngII-AT1 signaling pathways. Specifically, in human and 3T3-L1 preadipocytes, Ang(1–7)-Mas signaling promotes adipogenesis via activation of PI3K/Akt and inhibition of MAPK kinase/ERK pathways, and Ang(1–7)-Mas antagonizes the antiadipogenic effect of AngII-AT_1 by inhibiting the AngII-AT_1-triggered MAPK kinase/ERK pathway. The autocrine regulation of the AngII/AT_1-ACE2-Ang(1–7)/Mas axis upon adipogenesis has also been revealed. This study suggests the importance of the local regulation of the delicately balanced angiotensin system upon adipogenesis and its potential as a novel therapeutic target for obesity and related metabolic disorders.

The renin-angiotensin system (RAS)[2] critically regulates homeostasis in various body systems (1, 2). Angiotensin II (AngII), the major bioactive component of RAS, is converted from the precursor molecule angiotensinogen through two-step hydrolysis by renin and angiotensin-converting enzyme (ACE) (2). It acts through AngII type 1 (AT_1) and AngII type 2 (AT_2) receptors (3). AngII can be further hydrolyzed by ectoenzyme ACE2 into Ang(1–7) (4). Mediated by its interaction with the G-protein-coupled receptor Mas (Mas receptor) (5), Ang(1–7) usually antagonizes AngII actions (6).

RAS is also a crucial regulator of energy metabolism, implicated in metabolic disorders such as obesity and insulin resistance (7-9). Adipose tissue, a highly active metabolic and endocrine organ, is a source of components of the RAS (10, 11). On the other hand, AngII receptors and Mas receptors are co-expressed on adipocytes, implying the involvement of the local RAS system in regulating adipocyte functions (12, 13). Several lines of evidence have supported this emerging hypothesis. In particular, recent investigations have begun to reveal that ACE-AngII-AT_1 signaling and ACE2-Ang(1–7)-Mas signaling regulate various functions of adipocytes in an intriguing counteractive manner (6). For example, it has been demonstrated that AngII-AT_1 inhibits lipolysis and insulin-induced glucose uptake in adipocytes (14, 15), whereas Ang(1–7)-Mas does the opposite (16, 17). Also, AngII-AT_1 induces oxidative stress in adipocytes (18), whereas Ang(1–7)-Mas interaction suppresses it (17).

Previous studies have established the inhibitory effects of AngII-AT_1 on adipogenesis (19-21). We hypothesize that ACE2-Ang(1–7)-Mas acts as an autocrine balancer (feedback route) to promote adipogenesis. We aim, for the first time, to reveal the molecular mechanisms underlying Ang(1–7)-Mas regulation upon adipogenesis and its cross-talk with AngII-AT_1 pathways.

EXPERIMENTAL PROCEDURES

Cell Culture and Differentiation—Human subcutaneous preadipocytes (SP-F-2; Zen-Bio Inc.), collected from non-diabetic male subjects (with body mass index of 25–29.9), were grown until confluence (5% CO_2 and 37 °C) in preadipocyte

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[2] The abbreviations used are: RAS, renin-angiotensin system; AngII, angiotensin II; Ang(1–7), angiotensin 1–7; ACE, angiotensin-converting enzyme; AT_1, AngII type 1 receptor; AT_2, AngII type 2 receptor; Akt, protein kinase B; ACC, acetyl-CoA carboxylase; aP2, adipocyte protein 2; C/EBP, CCAAT/enhancer-binding protein; FAS, fatty acid synthase; PPAR, peroxisome proliferator-activated receptor; PMA, phorbol 12-myristate 13-acetate.

growth medium (DMEM/F-12 medium (1:1, v/v) containing 15 mM HEPES, 2 mM L-glutamine, 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 mg/ml streptomycin). Adipocyte differentiation was then induced (defined as day 0) similarly as reported previously (22, 23). Briefly, the cells were treated with adipocyte growth medium (serum-free DMEM/Ham's F-12 medium containing 15 mM HEPES, 2 mM L-glutamine, 33 μM biotin, 17 μM pantothenate, 10 μg/ml transferrin, 0.2 nM triiodothyronine, 100 units/ml penicillin, and 100 mg/ml streptomycin) supplemented with 0.5 μg/ml insulin, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 10 μM rosiglitazone. After 48 h (on day 2), the medium was replaced with adipocyte growth medium containing insulin and dexamethasone, and thereafter, the medium was refreshed every 2 days for the following 8–10 days.

The 3T3-L1 mouse preadipose cell line (American Type Culture Collection, Manassas, VA) was cultured in DMEM supplemented with 10% (v/v) bovine calf serum, 100 units/ml penicillin, 100 mg/ml streptomycin in a 5% CO_2 humidified atmosphere at 37 °C. Adipocyte differentiation of 3T3-L1 cells follows the previously established protocol (24, 25). Specifically, 2 days after the cells became confluent, adipocyte differentiation was induced (defined as day 0) by incubation with DMEM and 10% FBS, 5 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine. On day 2 of differentiation, DMEM containing 10% FBS and 5 μg/ml insulin was used as the culture medium. On day 4, the medium was then changed to DMEM with 10% FBS only, and thereafter, the medium was refreshed every 2 days for the following 2–4 days.

In some experiments, 10 μM LY294002, 50 μM PD98059, 100 nM PMA, 10 μM A779, 10 μM PD123319, 10 μM ZD7155, 1 μM AngII, or various concentrations of Ang(1–7) were added to the culture medium. In some experiments, a 30-min pretreatment of LY294002 or 60-min pretreatment of PD98059 was performed prior to the induction of differentiation. Ang(1–7), AngII, ZD7155, and PD123319 were purchased from Tocris Bioscience, and A779 was purchased from GenWay Biotech Inc. All cell culture media, supplements, and sera were purchased from Invitrogen. All other chemicals and reagents were purchased from Sigma-Aldrich.

siRNA Silencing—Gene silencing of Mas receptor (*Mas1*), ACE2 (*Ace2*), AT_1 (*Agtr1*), AT_2 (*Agtr2*), ERK1 (*Mapk3*), ERK2 (*Mapk1*), or Akt (*Akt1* and *Akt2*) in 3T3-L1 cells was achieved using a mouse MAS1 siRNA (sc62601, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)), a mouse ACE2 siRNA (sc-41401, Santa Cruz Biotechnology, Inc.), a mouse AT_1 siRNA (sc29751, Santa Cruz Biotechnology, Inc.), a mouse AT_2 siRNA (sc29753, Santa Cruz Biotechnology, Inc.), mouse ERK1 and ERK2 siRNAs (sc-29308 and sc-35336, Santa Cruz Biotechnology, Inc.), and a mouse Akt siRNA (sc-43610, Santa Cruz Biotechnology, Inc.), respectively. Transfection was done as described previously (26, 27). Specifically, 1 day after being split, the cells were incubated with Opti-MEM (Invitrogen) containing a complex of Lipofectamine-RNAiMAX transfection reagent (0.5% (v/v); Invitrogen) with siRNAs (20 nM) for 4 h, followed by the addition of an equal amount of DMEM containing 20% bovine calf serum and incubation for another 8–12 h. The cells were then maintained in DMEM containing 10% bovine calf serum until confluence, and adipocyte differentiation was initiated as described above.

Confocal Fluorescence Microscopy—Cells grown on glass coverslips were fixed with 3.7% formaldehyde in PBS for 15 min at room temperature and then washed with PBS three times. After being blocked with 1% bovine albumin (BSA) in PBST (PBS with 0.1% Tween 20) at room temperature for 1 h, the cells were incubated (at 4 °C) overnight with goat anti-Mas IgG in PBST with 1% BSA. After washing with PBS three times, the cells were incubated in FITC-labeled anti-goat IgG for 1 h at room temperature in the dark, followed by washing with PBS three times. Fluorescence signals were visualized on a confocal laser-scanning microscope (LSM 510 Meta, Carl Zeiss GmbH).

Western Blot Analysis—The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then scraped in radioimmune precipitation assay lysis buffer (sc-24948, Santa Cruz Biotechnology, Inc.) containing freshly added protease/phosphatase inhibitor mixture (Cell Signaling Technology). After centrifugation at 4 °C, the supernatant of the sample was collected, and its protein content was determined using a protein assay kit (Bio-Rad) based on the Bradford method. Each cell lysate with an equal amount of protein content (as the loading control) was separated on SDS-PAGE by electrophoresis, followed by transfer onto a nitrocellulose membrane. After blocking the membrane for 2–3 h in TBST (Tris-buffered saline-Tween; 10 mM Tris, 150 mM NaCl, and 0.05% Tween 20, pH 7.4) containing 5% BSA (Sigma), the membrane was incubated with primary antibodies (1:200–400 dilution) in TBST with 1% BSA overnight. After washing three times with TBST buffer, the membrane was subsequently incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000–3000 dilution) for 6–8 h. The protein bands were detected in a G:BOX Chemi XT4 imaging system (Syngene) by using Super-Signal West Pico chemiluminescent substrate (Thermo Scientific).

Antibodies against peroxisome proliferator-activated receptor γ (PPARγ) (sc7196), phospho-PPARγ (sc28001), C/EBPα (sc61), aP2 (A-FABP; sc18661), FAS (sc20140), AT_1 (sc31181), AT_2 (sc9040), MAS1 (sc54848), ACE2 (sc20998), Akt (sc8312), and phospho-Akt (Ser473) (sc135651) were purchased from Santa Cruz Biotechnology, Inc. Antibody against ACC (3662) was purchased from Cell Signaling Technology. Antibodies against phospho-ERK1/2 (3441-100) and ERK1/2 (3085-100) were purchased from Biovision Inc.

Oil Red O Staining—Lipid droplets in adipocytes were stained with Oil Red O (Sigma-Aldrich) as described previously (28). Briefly, after being fixed in 10% formalin for 60 min, the cells were washed twice with PBS and stained with filtered Oil Red O solution (1.8 mg/ml Oil Red O and 60% (v/v) isopropyl alcohol in distilled water) for 60 min. The cells were then thoroughly washed with distilled water before being photographed under an optical microscope.

Triglyceride Assay—Cellular triglyceride content was determined by using an adipogenesis assay kit (Biovision Inc.) according to the manufacturer's protocol. Briefly, after a 6–8-day differentiation of 3T3-L1 cells, or a 10–12-day differentiation of human preadipocytes, cells were washed twice with PBS, scraped, sonicated to homogenize the suspension, and then

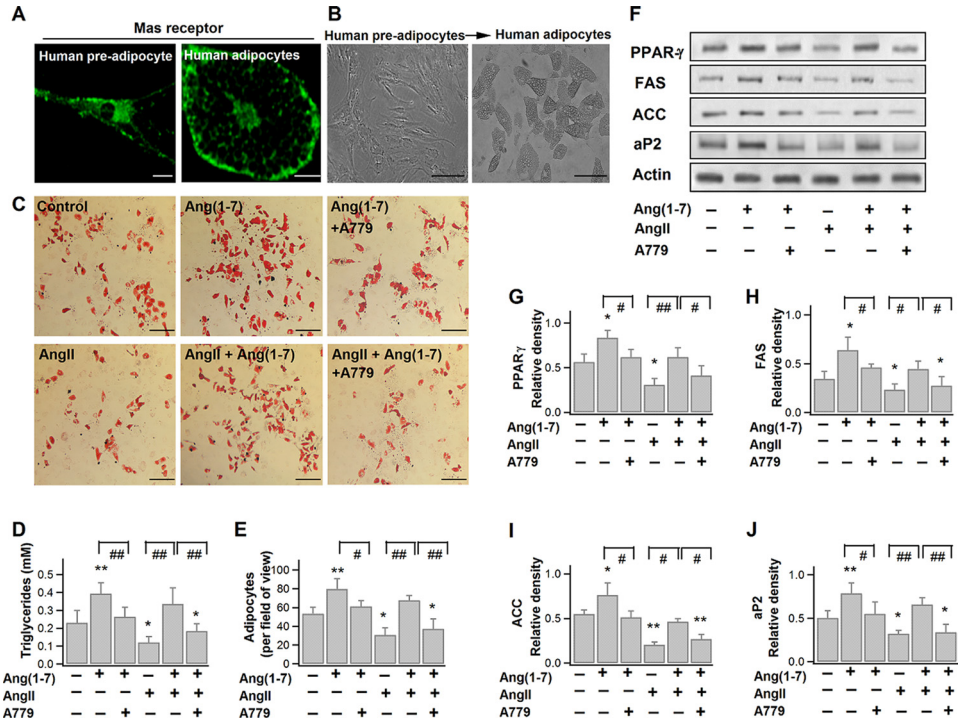

FIGURE 1. **Ang(1–7)-Mas receptor enhances adipogenesis and antagonizes the antiadipogenic effect of AngII in human preadipocytes.** *A*, confocal images of immunostained Mas receptors in human preadipocyte and adipocyte. *Scale bars*, 10 μm. *B*, representative optical images of human preadipocytes and differentiated human adipocytes (day 12). *Scale bars*, 60 μm. *C*, representative images of Oil Red O-stained adipocytes, differentiated from preadipocytes for 10–12 days without (control) or with exposure to 1 μM Ang(1–7), Ang(1–7) plus 10 μM A779 (specific Mas receptor antagonist), 1 μM AngII, AngII plus Ang(1–7), or AngII plus Ang(1–7) and A779. *Scale bars*, 240 μm. *D*, quantification of intracellular triglyceride content (mean ± S.E. (*error bars*), n = 4 - 5). *E*, number of adipocytes (lipid droplet-containing cells) per field of view (×40). The data are presented as mean ± S.E. (n = 6–7 images from 3–4 different culture batches). *F–J*, Western blot analyses of the expression of PPARγ (~54 kDa), FAS (~270 kDa), ACC (~280 kDa), aP2 (~15 kDa), and actin (~43 kDa) in differentiated human adipocytes (day 10–12 after induction of differentiation). *F*, representative immunoblots. *G–J*, statistics (mean ± S.E., n = 4 - 5) of the blot densities normalized to actin density (as the internal control). Each sample contains the same amount of total proteins (as the loading control). Student's t test was used: *, $p < 0.05$; **, $p < 0.01$ *versus* control; #, $p < 0.05$; ##, $p < 0.01$ between indicated pairs.

assayed for total triglyceride. The total protein concentration, which was used as an internal control, in these cells was measured with a protein assay kit from Bio-Rad.

Enzyme-linked Immunosorbent Assay (ELISA)—The concentration of Ang(1–7) or AngII in the medium was determined using a mouse Ang(1–7) ELISA kit (CSB-E13763m; Cusabio Biotech) or a mouse AngII ELISA kit (CSB-E04495m; Cusabio Biotech), respectively. Each sample contains the same amount of protein (determined by the Bradford method as the internal control of the total cell number).

Statistical Analysis—Data were analyzed using Student's t test and expressed as mean ± S.E. $p < 0.05$ was considered statistically significant.

RESULTS

Ang(1–7)-Mas Receptor Promotes Adipogenesis and Antagonizes the Antiadipogenic Effect of AngII-AT$_1$ Receptor in Human Preadipocytes—Preadipocytes isolated from human adipose tissue are used here as the *in vitro* cell model for adipogenesis (29, 30). It has been known that AngII receptors are expressed in preadipocytes, and the majority of AngII receptors found in human preadipocytes and adipocytes are of the AT$_1$ subtype (13, 31) (supplemental Fig. S1). Here, we report that Mas receptors are also expressed in human preadipocytes and adipocytes (Fig. 1*A* and supplemental Fig. S1). To evaluate the roles of Ang(1–7) and AngII in adipogenesis, human preadipocytes were induced to differentiate in the presence of Ang(1–7) and/or AngII.

Transformation of preadipocytes into mature adipocytes requires the coordinated regulation of various transcriptional factors (30). Among them, PPARγ is considered as the most essential one (32). Because adipogenesis is accompanied by lipogenesis, enzymes responsible for lipogenesis (*e.g.* fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC)) are also regarded as the important markers for adipogenesis (24, 33).

After 10–12 days of hormone-induced differentiation, many of the fibroblast-like spindle-shaped human preadipocytes were differentiated into mature adipocytes, as evidenced by their morphological change and intracellular accumulation of lipid droplets (Fig. 1*B*). Lipid accumulation, an indicator of the extent of adipogenesis, can be assessed visually by Oil Red O staining of lipid droplets and quantitatively by direct measurement of intracellular triglyceride (TG) content. As shown in Fig. 1, *C* and *D*, exogenous application of Ang(1–7) during differentiation considerably increased Oil Red O staining and TG content, and the number of lipid-laden adipocytes was also increased by Ang(1–7) treatment (Fig. 1*E* and supplemental Fig. S2*A*). Consistently, the addition of Ang(1–7) enhanced the expression of adipogenic transcriptional factor PPARγ, lipogenesis markers (FAS and ACC), and adipocyte protein-2 (aP2;

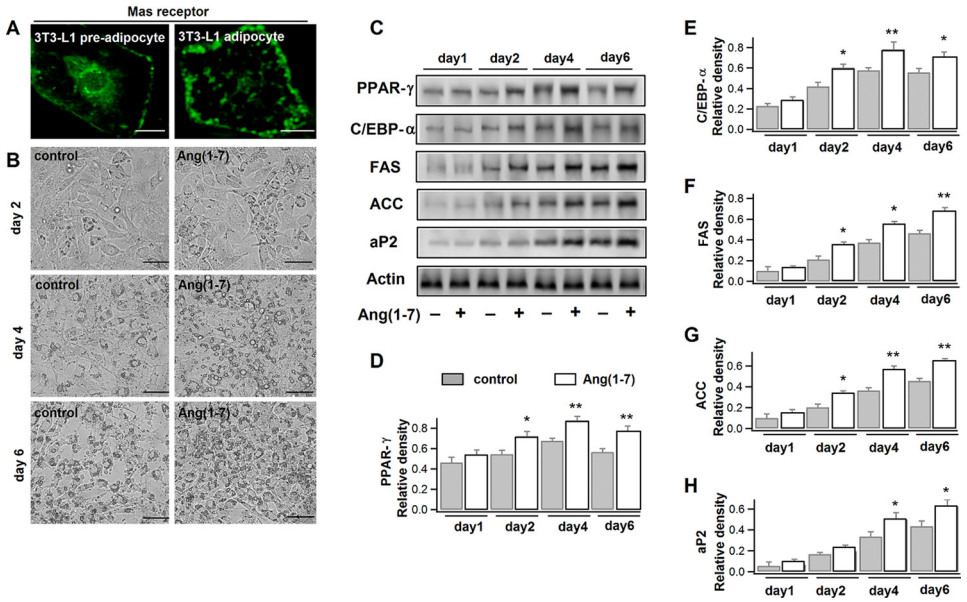

FIGURE 2. **Ang(1–7) enhances adipogenesis of 3T3-L1 preadipocytes.** *A*, confocal images of immunostained Mas receptor in 3T3-L1 preadipocyte and differentiated 3T3-L1 adipocyte (day 8). *Scale bars*, 10 μm. *B*, representative images of 3T3-L1 cells at different stages of differentiation (day 2, 4, and 6), without (control) or with exposure to Ang(1–7) *Scale bars*, 60 μm. *C–H*, Western blot analyses of PPARγ, C/EBPα (~42 kDa), FAS, ACC, aP2, and actin expression in 3T3-L1 cells at different stages of differentiation (day 1, 2, 4, and 6), without (−) (control) or with (+) 1 μM Ang(1–7). Representative immunoblots are shown in *C*, and the statistics (mean ± S.E. (*error bars*), $n = 4–5$) of the optical density ratio normalized to the actin blot (internal control) are shown in *D–H*. Each sample contains the same amount of total protein (loading control). Student's *t* test was used: *, $p < 0.05$; **, $p < 0.01$ *versus* control.

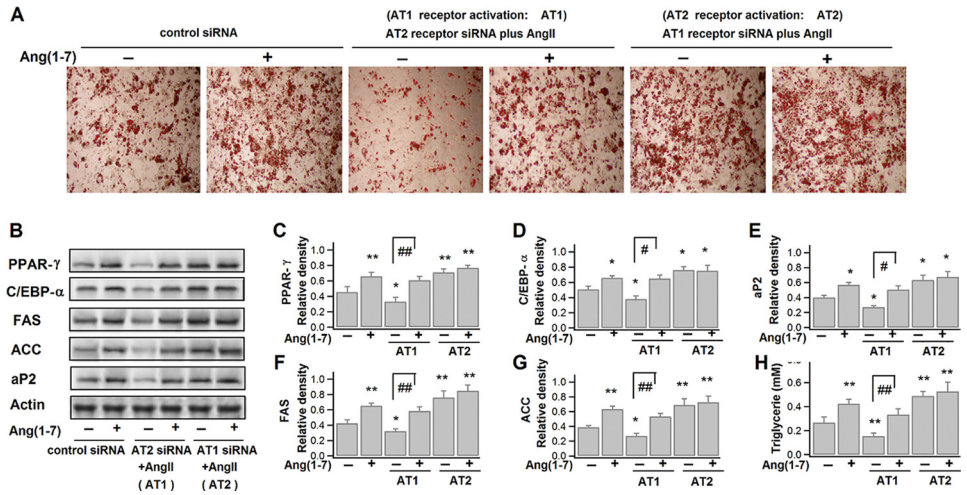

FIGURE 3. **Ang(1–7) stimulates adipogenesis and neutralizes the antiadipogenic effect of AngII-AT$_1$ receptor activation in 3T3-L1 preadipocytes.** The cells were transfected with control siRNA or AT$_1$ receptor siRNA or AT$_2$ receptor siRNA and cultured until confluence. This was followed by induction of differentiation for 7–8 days, without (−) (control) or with (+) 1 μM Ang(1–7) and/or 1 μM AngII. *A*, representative images of Oil Red O-stained 3T3-L1 adipocytes differentiated under different conditions (day 8). *B–G*, Western blot analyses of the expression of PPARγ, C/EBPα, FAS, ACC, aP2, and actin in 3T3-L1 adipocytes (day 7–8). Representative immunoblots are shown in *B*. The statistics (mean ± S.E., $n = 5–6$) of the optical densities of the blots normalized to that of actin are shown in *C–G*. *H*, intracellular triglyceride content (mean ± S.E., $n = 4–5$) in 3T3-L1 adipocytes (day 7–8). Student's *t* test was used: *, $p < 0.05$; **, $p < 0.01$ *versus* control; #, $p < 0.05$; ##, $p < 0.01$ between indicated pairs.

a specific late marker of adipogenesis (24)) (Fig. 1, *F–J*). Clearly, Ang(1–7) stimulates the adipocytic differentiation of human preadipose cells.

It has been reported that AngII suppresses adipogenesis of human preadipocytes via the AT$_1$ receptor (19, 20). As expected, the addition of AngII during differentiation suppressed adipogenesis, as evidenced by reduced Oil Red O staining, TG content, the number of adipocytes, and the expression of adipogenesis markers (PPARγ, FAS, ACC, and aP2) (Fig. 1). Such AngII-induced suppression is mediated by AT$_1$ receptors because it can be rectified by a specific AT$_1$ antagonist (ZD7155) (34) but not by a specific inhibitor of AT$_2$ (PD123319) (35) (supplemental Fig. S2*B*).

Interestingly, co-incubation with Ang(1–7) effectively abolished the antiadipogenic effects of AngII (Fig. 1). To determine if Ang(1–7) mediates its stimulatory actions through the Mas receptor, the selective Mas receptor antagonist A779 (36) was applied during differentiation. As shown in Fig. 1, the addition of A779 completely eliminated the stimulatory effects of Ang(1–7) on adipogenesis and its counteraction on AngII. Taken together, we conclude that Ang(1–7), via its interaction with the Mas receptor, not only

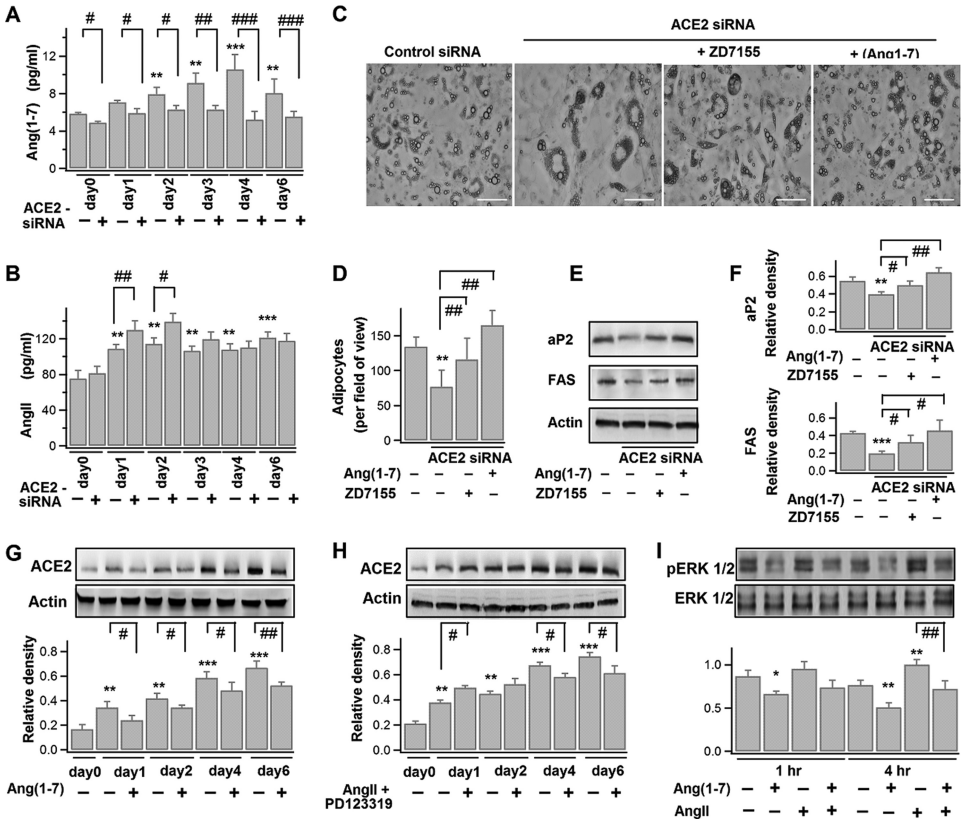

FIGURE 4. **Autocrine interplays between Ang(1–7) and AngII in the differentiation of 3T3-L1 preadipocytes.** The cells were transfected with control siRNA or ACE2 siRNA, and cultured until confluence. This was followed by 7–8 days of differentiation, with (+) or without (−) 1 μM Ang(1–7) or 10 μM ZD7155 (selective AT_1 receptor antagonist) or 1 μM AngII plus PD123319 (selective AT_2 receptor antagonist). *A* and *B*, the concentrations of released Ang(1–7) and AngII in the culture media (24 h incubation) at different differentiation stages (day 0–6). The concentrations at day 0 from the control siRNA-transfected cells were regarded as the control). *C*, representative images of differently cultured 3T3-L1 cells (day 7–8). *Scale bars*, 60 μm. *D*, number of adipocytes (lipid droplet-laden cells) per field of view (×40). The data is presented as mean ± S.E. (*error bars*) (n = 6–7 images from 3–4 different culture batches). *E* and *F*, representative immunoblots of aP2, FAS, and actin (day 7–8) and the statistics (mean ± S.E., n = 4–5) showing the optical densities of the blots normalized to actin density. *G* and *H*, Western blot analyses of ACE2 (~90 kDa) and actin expression in untreated 3T3-L1 cells at different differentiation stages (day 0–6) (expression level at day 0 as control). The *top panel* shows the representative immunoblots, and the *bottom panel* shows the statistics (mean ± S.E., n = 4–5) of the optical density of the ACE2 normalized to that of actin. *I*, Western blot analyses of the phosphorylated and total ERK1/2 (pERK1/2 and ERK1/2, ~42–44 kDa) in AT_2 siRNA-transfected cells (1 or 4 h after induction of differentiation). The *top panels* show the representative immunoblots, and the *bottom panel* shows the statistics (mean ± S.E., n = 4–5) of the optical density ratio between pERK1/2 and ERK1/2. Student's t test was used: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *versus* control; #, $p < 0.05$; ##, $p < 0.01$ between indicated pairs.

stimulates adipogenesis but also counteracts the antiadipogenic effect of AngII.

The Adipogenic Effect of Ang(1–7) and Its Counteraction against AngII-AT_1 Activation Are Confirmed in 3T3-L1 Preadipocytes—Because human preadipocytes grow and differentiate slowly and cannot be easily transfected (37), we used an established mouse preadipocyte cell line (3T3-L1 cells) (29, 30) instead as the cell model in all of the following experiments in order to investigate the cross-talk between Ang(1–7) and AngII signaling observed from human preadipocytes. We first confirmed that 3T3-L1 cells express Ang(1–7) receptor (Mas receptor) and AngII receptors (both AT_1 and AT_2 receptors) (Fig. 2*A* and supplemental Fig. S1) (38, 39).

As 3T3-L1 preadipocytes were differentiating into adipocytes (day 0–6 after hormonal induction of differentiation), the expression of PPARγ, C/EBPα, FAS, ACC, and aP2 were significantly up-regulated, along with their morphological change and increased intracellular accumulation of lipid droplets (Fig. 2). Consistent with the observations from human preadipocytes (Fig. 1), Ang(1–7) treatment during differentiation increased the expression of adipogenic markers (PPARγ, C/EBPα, FAS, ACC, and aP2), Oil Red O staining, and TG content (Fig. 2 and supplemental Fig. S3). Ang(1–7)-stimulated adipogenesis is apparently dose-dependent (supplemental Fig. S3).

In order to evaluate the role of AngII in adipogenesis of 3T3-L1 cells, we have to differentiate the effects mediated by the AT_1 or AT_2 receptor. This is achieved by challenging the AT_2 siRNA knockdown 3T3-L1 cells or AT_1 siRNA knockdown cells with AngII. Knockdown of the AT_1 or AT_2 gene (*Agtr1* or *Agtr2*) was confirmed by Western blot analyses (supplemental Fig. S1). As shown in Fig. 3, activation of AT_1 receptors decreased Oil Red O staining, TG content, and expression of all adipogenic markers (PPARγ, C/EBPα, FAS, ACC, and aP2), whereas AT_2 activation did the opposite. These results were corroborated in naive 3T3-L1 cells by selective inhibition of AT_1 or AT_2 using their specific antagonists (supplemental Fig. S4). These experiments establish the inhibitory role of AngII-AT_1 signaling in adipogenesis, which is in agreement with previous reports (19–21) and our observations in human preadipocytes (Fig. 1). Also similar to the observations from human preadipocytes (Fig. 1), co-incubation of Ang(1–7) essentially

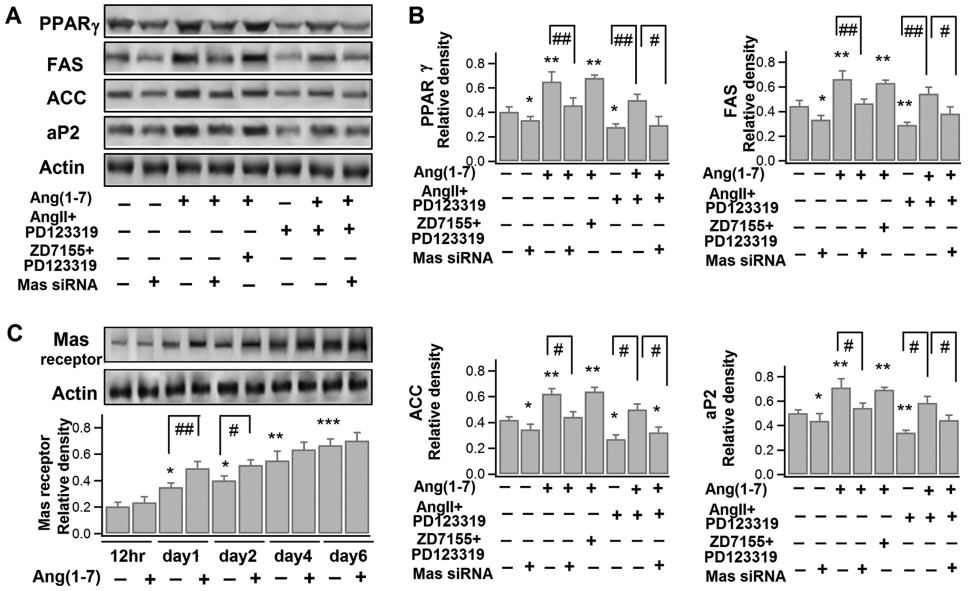

FIGURE 5. **Involvement of Mas receptor in 3T3-L1 adipogenesis.** The cells were transfected with control siRNA or Mas receptor siRNA and cultured until confluence. Differentiation was then induced for 7–8 days, without (−) (control) or with (+) 1 μM Ang(1–7), 1 μM AngII, 10 μM ZD7155, or 10 μM PD123319. *A* and *B*, Western blot analyses of PPARγ, FAS, ACC, aP2, and actin (day 7–8). The representative immunoblots are shown in *A*, and the statistics (mean ± S.E. (*error bars*), $n = 4–5$) of the optical densities of the blots normalized to actin density are shown in *B*. *C*, Western blot analyses of Mas receptor (~37 kDa) and actin in the untreated cells at different stages of differentiation (12 h to day 6). Protein level at 12 h without Ang(1–7) treatment was regarded as the control. The *top panel* shows the representative immunoblots, and the *bottom panel* shows the statistics (mean ± S.E., $n = 4–5$) of the optical density ratio between the blot of Mas receptor and that of actin. Student's *t* test was used: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *versus* control; #, $p < 0.05$; ##, $p < 0.01$ between indicated pairs.

reversed the inhibitory effects of AT_1 receptor activation upon adipogenesis. On the other hand, Ang(1–7) did not affect AT_2-stimulated adipogenesis (Fig. 3 and supplemental Fig. S4). Taken together, we show that 3T3-L1 is a good and convenient alternative model to human preadipocytes to study the stimulatory roles of Ang(1–7)-Mas signaling in adipogenesis and its cross-talk with AngII-AT_1 pathways.

Autocrine Interplays between Ang(1–7) and AngII—It is known that production of AngII and its precursor peptide (angiotensinogen) increases during adipogenesis (20, 40). AngII peptides, in turn, are cleaved by a membrane-bound ectoenzyme (ACE2) to form Ang(1–7) (4). It is thus conceivable that Ang(1–7) production might increase as well during adipogenesis. Indeed, as shown in Fig. 4, *A* and *B*, both Ang(1–7) and AngII released into the culture media were elevated during adipogenesis. Because the receptors for Ang(1–7) and AngII are expressed in preadipocytes and adipocytes (12, 39) (Figs. 1 and 2 and supplemental Fig. S1), it is likely that these locally produced Ang(1–7) and AngII may serve as the autocrine feedback signals upon adipocyte differentiation in an interactive way.

Ace2 gene expression in 3T3-L1 cells was blocked by specific ACE2 siRNA before the initiation of differentiation (verified by Western blot analyses shown in supplemental Fig. S1). As a result, it was found that the release of Ang(1–7) from these cells was considerably lowered throughout the differentiation, whereas the AngII release was increased in the early stage (day 1–2) (Fig. 4, *A* and *B*). It was also noted that adipogenesis was suppressed in ACE2 siRNA knockdown cells, as evidenced by the reduced number of lipid-laden adipocytes and decreased expression of adipocyte markers: aP2 and FAS (Fig. 4, *C–F*). This is presumably due to the decrease of the adipogenic factor Ang(1–7) and increase of the antiadipogenic factor AngII. In support of this, we show that down-regulation of adipogenesis by ACE2 knockdown can be effectively rectified by application of Ang(1–7) or blocking of the AT_1 receptor. These results demonstrate that ACE2 is a critical linker to control the balance between Ang(1–7) and AngII signaling.

Fig. 4, *G* and *H*, shows that ACE2 expression steadily increased over time during the 3T3-L1 differentiation, promoting adipogenesis by strengthening Ang(1–7) signaling. Intriguingly, exogenous application of Ang(1–7) suppressed the increase of ACE2 expression (Fig. 4*G*), whereas AngII-AT_1 activation further enhanced ACE expression in the early differentiation phase (day 1). These observations suggest that a delicate balance is established by the interplays between AngII, ACE2, and Ang(1–7).

It is known that the antiadipogenic effect of AngII-AT_1 involves the activation (phosphorylation) of ERK1/2 (19). Here, we show that Ang(1–7) neutralizes the antiadipogenic effect of AngII-AT_1 by interfering with ERK1/2 activation because, in AT_2 siRNA knockdown cells, AngII-enhanced ERK1/2 phosphorylation was abolished by co-incubation of Ang(1–7) (Fig. 4*I*). In summary, we provide evidence that the local autocrine regulations of Ang(1–7) and AngII play important and counteracting roles in adipogenesis, and the cross-talks between Ang(1–7)-Mas and AngII-AT_1 signaling are linked by ACE2 and ERK1/2.

Involvement of Mas Receptor—The experiments on human preadipocytes (Fig. 1) demonstrate that the adipogenic and anti-AngII effects of Ang(1–7) are mediated by the Mas receptor. Here, we further analyzed the involvement of the Mas receptor using *Mas1* gene knockdown 3T3-L1 cells. The knockdown of Mas receptor expression by MAS1 siRNA was verified by Western blot analyses (supplemental Fig. S1).

In the Mas knockdown cells, the expressions of adipogenic markers (PPARγ, FAS, ACC, and aP2) were reduced (Fig. 5, *A*

and *B*), and as expected, Ang(1–7) failed to increase the expression of these markers and to abolish the inhibition by AngII. Some studies have reported low affinity interaction of Ang(1–7) with AT_1 and AT_2 receptors in other cell types (41, 42). To rule out the possible contributions from AT_1 and AT_2 receptors, we show that Ang(1–7)-stimulated expression of the adipogenic markers was not compromised when AT_1 and AT_2 receptors were simultaneously blocked by their respective antagonists. These results establish that the adipogenic effects of Ang(1–7) are mediated by Mas receptors.

Furthermore, we also observed that Mas receptor expression increased over time during the 3T3-L1 differentiation, and application of exogenous Ang(1–7) elevated Mas receptor expression at the early differentiation phase (day 1–2) (Fig. 5*C*). These experiments demonstrate the positive regulation of Ang(1–7)-Mas signaling to accelerate adipogenesis, particularly in the early stage.

Stimulation of Adipogenesis by Ang(1–7) is MAPK Kinase/ERK- and PI3K/Akt-dependent—Next, we explored the signaling pathways responsible for the stimulatory effect of Ang(1–7) on adipogenesis of 3T3-L1 cells. It is known that the phosphorylation (activation) of ERK1/2 is required for mitotic clonal expansion in the early stage of adipogenesis (43, 44); however, ERK1/2 activity must quickly return to a low level to avoid inactivation (phosphorylation) of the critical adipogenic factor (PPARγ) (45, 46). Indeed, as shown in Fig. 6*A*, hormonal stimulation of adipogenesis caused an acute increase of ERK1/2 phosphorylation (within 15 min), which was followed by a quick decay to a low level. Interestingly, such decay was exaggerated by the addition of Ang(1–7) (Fig. 6*A*). As discussed above, it would help to sustain the adipogenic process by preventing ERK1/2-induced inactivation (phosphorylation) of PPARγ. This is confirmed in Fig. 6*B*.

PI3K/Akt signaling-triggered PPARγ expression is also well known for its critical role in adipogenesis (47, 48). Regulation of Ang(1–7) on the PI3K/Akt pathway has been reported in other cell types (49, 50). As shown in Fig. 6*C*, phosphorylation of Akt was acutely induced (within 5 min) upon induction of adipogenic differentiation, and this acute phosphorylation was largely enhanced by the addition of Ang(1–7), although such enhancement is short lived (within 15 min).

ERK1/2 and Akt are the substrates of MAPK kinase and PI3K, respectively. Stimulation of MAPK kinase by phorbol 12-myristate 13-acetate (PMA) produced a significant inhibitory effect on adipogenesis, as evidenced by the reduced number of mature adipocytes and expression of adipogenic markers (aP2, FAS, ACC, and PPARγ) (Fig. 7, *A–G*). On the contrary, inhibiting MAPK kinase by PD98059 (51) slightly enhanced adipogenesis (Fig. 7 and supplemental Fig. S5). PD98059 inhibition and PMA stimulation upon adipogenesis have also been reported previously (52–54). Here, we further show that PMA was able to partially eliminate Ang(1–7)-induced adipogenesis (Fig. 7 and supplemental Fig. S5). In another line of experiments, it was found that blocking PI3K by LY294002 (55) significantly inhibited adipogenesis and abolished the stimulatory effect of Ang(1–7) (Fig. 7, *A–G*).

The ERK1/2 inhibition and Akt stimulation upon adipogenesis were further confirmed by knockdown of ERK1/2 (*Mapk3* and *Mapk1*) or *Akt1/2* genes before the initiation of 3T3-L1 cell differentiation. The knockdown of the protein expression was verified by Western blot (supplemental Fig. S5). In the ERK1/2 knockdown cells, the expressions of adipogenic markers (aP2 and FAS) were significantly increased, whereas in the Akt knockdown cells, the expressions of these markers were largely suppressed (Fig. 7, *H–J*). Also, as expected, Ang(1–7) failed to increase the expressions of these markers in the Akt knockdown cells (Fig. 7, *H–J*).

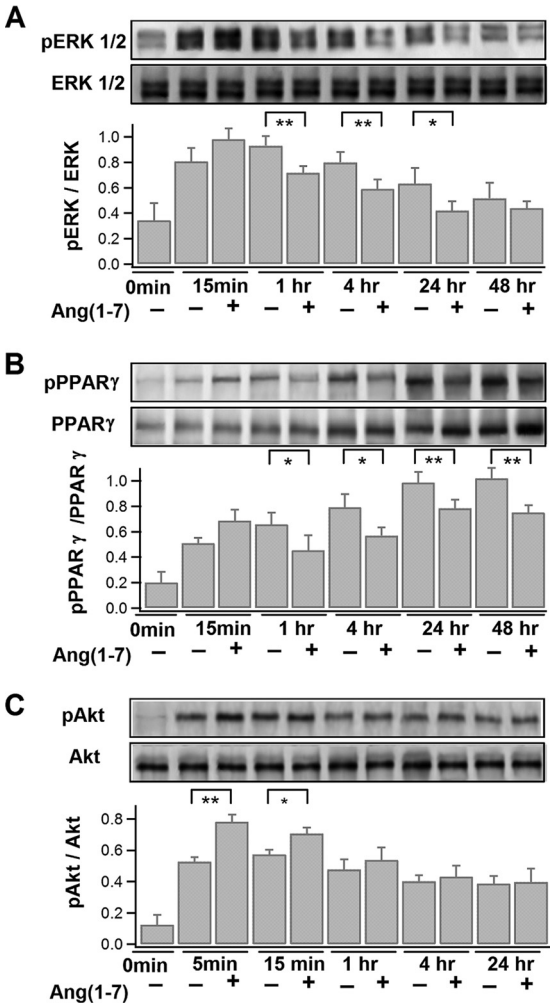

FIGURE 6. **Effects of Ang(1–7) on the phosphorylation of ERK1/2, PPARγ and Akt in 3T3-L1 cells during the early stage of differentiation.** Shown are Western blot analyses of the phosphorylation and total protein levels of ERK1/2 (*A*), PPARγ (*B*), and Akt (~60 kDa) (*C*) in 3T3-L1 cells at different times of differentiation (0 min, 5 min, 15 min, 1 h, 4 h, 24 h, and 48 h), with (+) or without (−) 1 μM Ang(1–7) treatment. The *top panels* show the representative immunoblots. The *bottom panels* show the statistics (mean ± S.E. (*error bars*), $n = 5–6$) of the optical density ratio between phospho-ERK1/2 (*pERK1/2*) and ERK1/2 blots, between phospho-PPARγ (*pPPARγ*) and PPARγ blots, or between phospho-Akt (*pAkt*) and Akt blots. Student's *t* test was used: *, $p < 0.05$; **, $p < 0.01$ between indicated pairs.

Taken together, it may be concluded that Ang(1–7) stimulates adipogenesis through two distinct pathways: 1) inhibiting MAPK kinase-ERK1/2 signaling, which leads to PPARγ inactivation, and 2) enhancing PI3K/Akt signaling, which leads to PPARγ expression. The latter pathway appears to be more potent.

Ang(1–7) Acts in the Early Stage of Differentiation—The acute action of Ang(1–7) on ERK1/2 and Akt phosphorylation

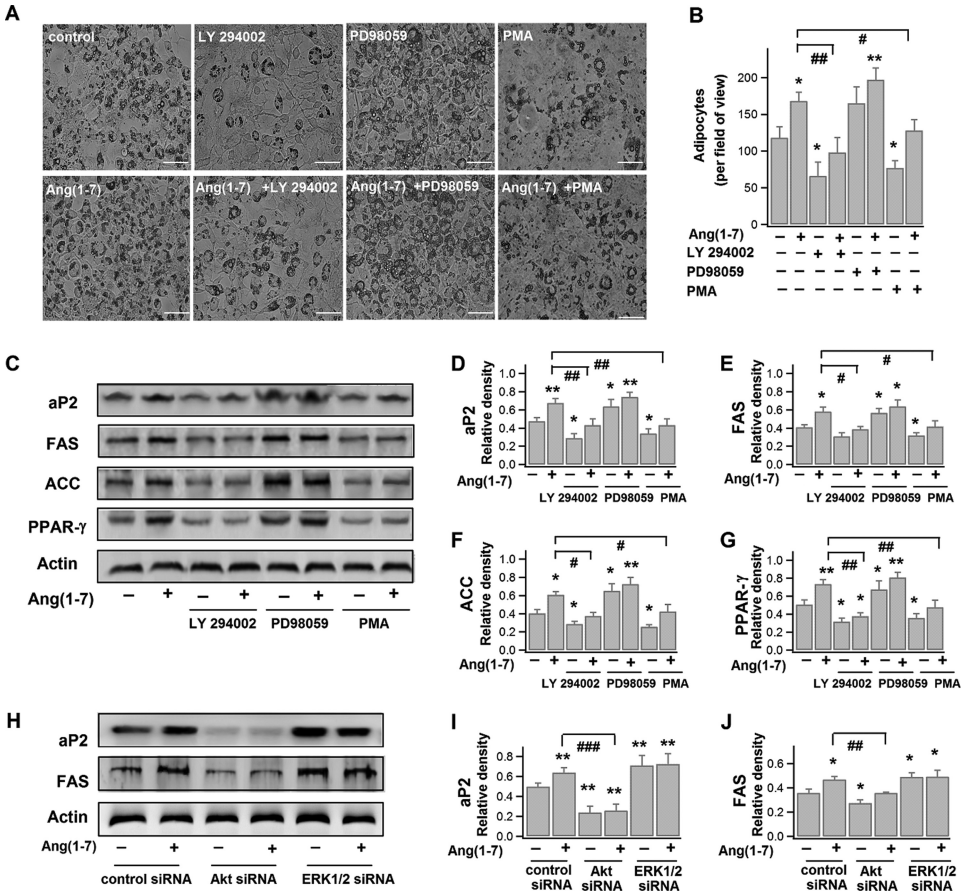

FIGURE 7. **Effect of PI3K/Akt and MAPK kinase/ERK signaling pathways on Ang(1–7)-induced adipogenesis of 3T3-L1 cells.** The cells were induced to differentiate for 7–8 days without (control) or with 1 μM Ang(1–7), 10 μM LY294002 (selective inhibitor of PI3K), Ang(1–7) plus LY29400, 50 μM PD98059 (selective inhibitor of MAPK kinase), Ang(1–7) plus PD98059, 100 nM PMA, or Ang(1–7) plus PMA. *A*, representative images of differently cultured cells (day 7–8). *Scale bars*, 60 μm. *B*, the number of adipocytes (lipid droplet-containing cells) per field of view (×40) was counted on day 7 and is presented as mean ± S.E. (*error bars*) ($n = 6$–7 images from 3–4 different culture batches). *C–G*, Western blot analyses of aP2, FAS, PPARγ, ACC, and actin expression in 3T3-L1 cells (day 7–8). Representative immunoblots are shown in *C*, and the statistics (mean ± S.E., $n = 5$–6) of the optical density ratio normalized to the actin blot are shown in *D–G*. *H–J*, 3T3-L1 cells were transfected with control siRNA or Akt siRNA or ERK1/2 siRNA and cultured until confluence. Differentiation was then induced for 7–8 days, without (−) (control) or with (+) 1 μM Ang(1–7). Representative immunoblots of aP2, FAS, and actin expression in 3T3-L1 cells (day 7–8) are shown in *H*, and the statistics (mean ± S.E., $n = 4$–5) of the optical density ratio normalized to the actin blot are shown in *I* and *J*. Student's *t* test was used: *, $p < 0.05$; **, $p < 0.01$ *versus* control; #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ between indicated pairs.

(shown in Fig. 6) suggests the importance of Ang(1–7) signaling in the early stage of differentiation. In support of this view, it was found, based on the number of differentiated adipocytes and expressions of adipogenic markers (aP2 and FAS), that administration of Ang(1–7) even 1 day after the hormonal initiation of differentiation failed to stimulate adipogenesis, whereas incubation with Ang(1–7) only for the first day of differentiation was as potent as its presence for the whole differentiation process (6 days) (Fig. 8).

To further confirm that Ang(1–7) acts mainly on the early stage of differentiation, the Mas receptor was knocked down at different stages of differentiation (at day −2 (2 days before the initiation of differentiation) or at day 0, day 2, or day 4) (Fig. 8, *G–I*). The Mas receptor expression was largely suppressed even 1 day after the transfection with MAS1 siRNA, which was verified by Western blot analyses (supplemental Fig. S1). Consistent with our data shown in Fig. 5, Ang(1–7) failed to increase the expression of adipogenic markers (aP2 and FAS) in the 3T3-L1 cells transfected with MAS1 siRNA at day −2; however, Ang(1–7) was able to significantly stimulate the expressions of these markers in the cells transfected with MAS1 siRNA at day 0, day 2, or day 4 (Fig. 8, *G–I*).

It is known that, in the early stage of differentiation, preadipocytes undergo several rounds of cell division, known as mitotic clonal expansion, before undertaking terminal differentiation, through which they acquire the characteristics of maturity (33, 43). We show in Fig. 8*C* that induction of differentiation resulted in 2–3-fold increase in cell number within 2 days, but Ang(1–7) treatment had an insignificant effect on cell proliferation. This observation rules out the possibility that the adipogenic effects of Ang(1–7) is due to increased mitotic clonal expansion.

DISCUSSION

Adipocytes, which were previously regarded as the passive storage depots for excess energy (fat), actually play perplexing and integrative roles in regulating metabolism (56). Complicated cross-talks exist between adipocytes and other cells in the metabolic networks (57). For example, the functions of adipocytes (adipogenesis, lipid, and carbohydrate metabolism) are

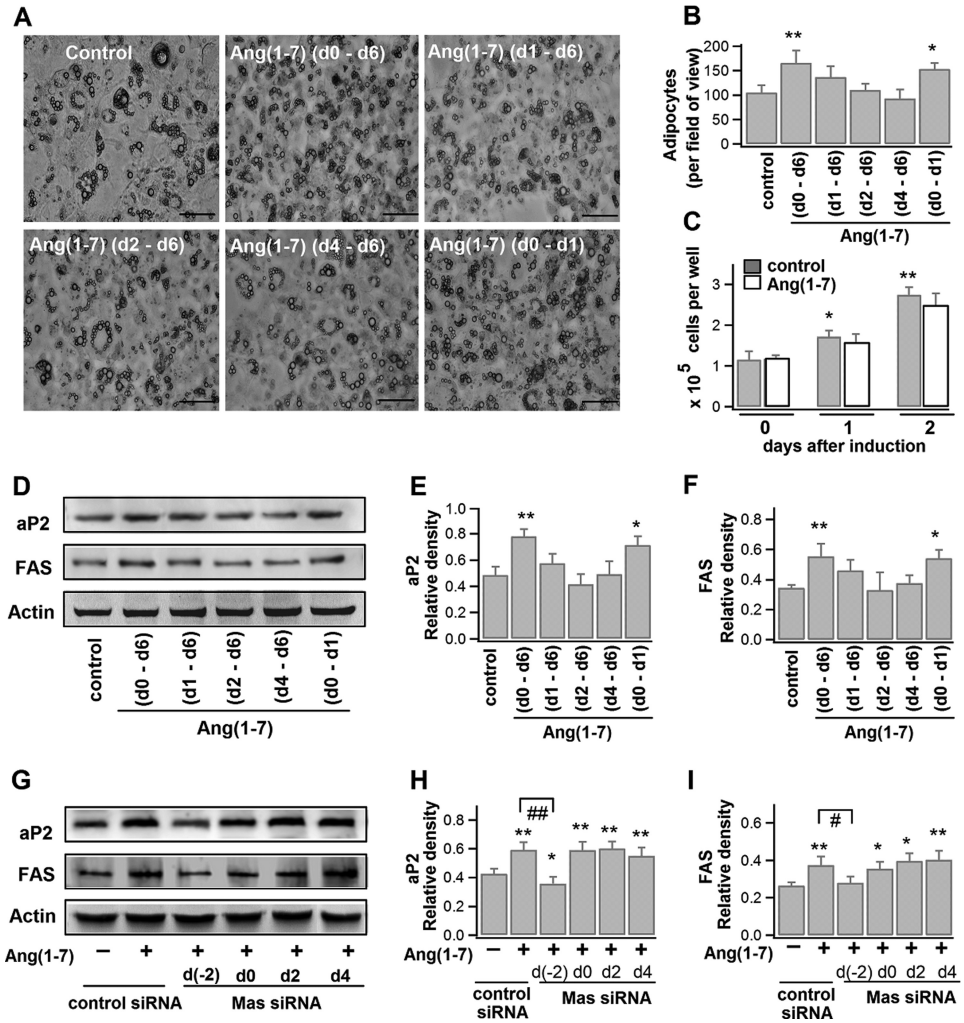

FIGURE 8. **Ang(1–7) exerts its stimulatory effect on adipogenesis in the early stage of differentiation.** 3T3-L1 cells were induced to differentiate without (control) or with exposure to 1 μM Ang(1–7) for different periods of time: from day 0 to 6 (*d0-d6*), from day 1 to 6 (*d1–d6*), from day 2 to 6 (*d2–d6*), from day 4 to 6 (*d4 – d6*), or from day 0 to 1 (*d0 – d1*). *A*, representative images of differently cultured 3T3-L1 cells (day 6). *Scale bars*, 60 μm. *B*, the number of adipocytes (lipid droplet-containing cells) per field of view (×40) was counted on day 6 and is presented as mean ± S.E. (*error bars*) ($n = 6–8$ images from 3–4 different culture batches). *C*, number of 3T3-L1 cells per 12 wells was counted at days 0, 1, and 2 (mean ± S.E., $n = 4$; the cell number at day 0 was regarded as the control). *D–F*, Western blot analyses of aP2, FAS, and actin expression in differently cultured cells (day 6). Representative immunoblots are shown in *D*, and the statistics (mean ± S.E., $n = 4–5$) of the optical density ratio normalized to the actin blot are shown in *E* and *F*. *G–I*, 3T3-L1 cells were transfected with control siRNA or Mas receptor siRNA at different times. Shown is transfection of Mas receptor siRNA 2 days before the initiation of differentiation (*d(−2)*) and at day 0 (*d0*), day 2 (*d2*), and day 4 (*d4*). Differentiation was induced at day 0 without (−) (control) or with (+) 1 μM Ang(1–7) for 7–8 days. Representative immunoblots of aP2, FAS, and actin expression in 3T3-L1 cells (day 7–8) are shown in *G*, and the statistics (mean ± S.E., $n = 4–5$) of the optical density ratio normalized to the actin blot are shown in *H* and *I*. Student's *t* test was used: *, $p < 0.05$; **, $p < 0.01$ *versus* control; #, $p < 0.05$; ##, $p < 0.01$ between indicated pairs.

highly regulated by insulin secreted by pancreatic beta cells (58), catecholamines secreted by chromaffin cells (59), and glucocorticoid secreted by adrenal cortex (60). On the other hand, adipocytes release a variety of adipokines (*e.g.* leptin, apelin, and AngII) to control various aspects of metabolism and influence the secretion of other metabolic factors or hormones from other cells (59, 61). Intriguingly, the receptors of some adipokines are identified in adipocytes, implying the existence of autocrine regulatory loops on adipocyte functions (39, 62, 63). For instance, adiponectin-adiponectin receptor (AdipoR1 and AdipoR2) promotes adipogenesis and insulin-induced glucose uptake and inhibits lipolysis (64, 65); leptin-leptin receptor (ObR) inhibits adipogenesis and glucose uptake and promotes lipolysis (54, 66); and apelin-apelin receptor inhibits adipogenesis and lipolysis (26) and promotes insulin sensitivity and glucose uptake (67).

In this study, we demonstrate the local autocrine regulation by the angiotensin system. Specifically, the counteracting interplays between Ang(1–7)-Mas and AngII-AT_1 signaling upon adipogenesis are revealed, as illustrated in Fig. 9, Based on our experiments and the previous reports (5, 19, 45, 48), the following scenario may be proposed. During adipogenesis, the production of AngII (Fig. 4) and AT_1 receptor is up-regulated (20, 40), acting as a dampening feedback (Figs. 1 and 3). Local accumulation of AngII stimulates the expression of ACE2 and production of Ang(1–7) by ACE2 (Fig. 4). Mediated by Mas receptor, Ang(1–7), in turn, enhances adipogenesis (Figs. 1–5) by inhibiting the AngII-AT_1-triggered MAPK kinase-ERK1/2 pathway to activate the adipogenic factor PPARγ (Figs. 4, 6, and 7) and by stimulating the PI3K/Akt pathway to increase PPARγ expression (Figs. 6 and 7). This action is further augmented by Ang(1–7)-induced Mas receptor expression (Fig. 5). Ang(1–7),

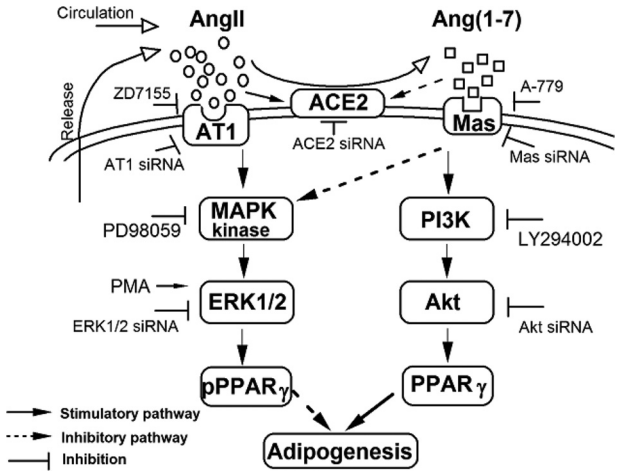

FIGURE 9. **Illustration of the interplays between Ang(1–7)-Mas and AngII-AT_1 signaling in adipogenesis.**

on the other hand, reduces ACE2 expression presumably to avoid overproduction of Ang(1–7) (Fig. 4). As described, multilateral negative and positive feedback loops peculiarly co-exist. Presumably, such complex interplays are balanced to ensure appropriate pace and phasing in differentiation. Disruption of these subtle interplays by silencing ACE2 expression or Mas receptor expression freezes adipogenesis, whereas the addition of exogenous Ang(1–7) accelerates adipogenesis.

It is increasingly accepted that formation of new adipocytes (adipogenesis) is a physiological adaptive response to excessive calorie intake in order to maintain an overall healthier adipose tissue because newly differentiated adipocytes are more insulin-sensitive (thus more capable of energy storage) (68, 69). Otherwise, hypertrophic adipocytes, which are less insulin-sensitive, will result (70, 71). The adverse metabolic consequences of enlarged adipocytes (particularly in visceral fat) have been extensively documented, including insulin resistance-caused type 2 diabetes and hypertriglyceridemia (72, 73). The adipogenic potential of preadipocytes from different fat depots differs. Subcutaneous preadipocytes are more sensitive to the adipogenic stimuli (*e.g.* PPARγ agonist) than visceral ones (74, 75). Adipogenesis in subcutaneous adipose tissues thus assists in preventing the notorious hypertrophy of visceral adipose (76). Therefore, the adipogenic effect of Ang(1–7) signaling should, albeit counterintuitively, be beneficial. This is supported by previous studies on animal models. For instance, increasing the circulating level of Ang(1–7) in rats improves insulin sensitivity in adipose tissue and decreases the plasma level of triglyceride and visceral fat mass (7, 77), whereas knockdown of Mas receptors in mice results in impaired insulin sensitivity and increased visceral fat mass (78). In contrast, the antiadipogenic AngII-AT_1 signaling increases visceral adipose tissue growth and insulin resistance (19, 79, 80), whereas administration of AT_1 receptor blockers reduces fat mass and improves insulin sensitivity in human and several animal models (81–83).

Through circulation, the autocrine balance between AngII and Ang(1–7) signaling can be influenced by other angiotensin-peptide producing cells (*e.g.* vascular endothelial and smooth muscle cells, cardiac myocytes, pancreatic beta cells, hepatocytes, etc.) (2, 6). On the other hand, adipose tissue is a major source of angiotensin peptides (10, 84), which play critical roles in regulating, for example, blood vessel contraction (85), angiogenesis (86), glucose uptake in skeletal muscles (9), and hormone secretion (39, 87). Such cross-talks may underlie the association between obesity and other metabolic disorders (*e.g.* diabetes and cardiovascular diseases). Hence, this study not only reveals the intriguing autocrine interplays between AngII and Ang(1–7) signaling upon adipogenesis but also raises the possibility of targeting these molecular pathways to treat metabolic disorders.

REFERENCES

1. Cleland, S. J., and Reid, J. L. (1996) The renin-angiotensin system and the heart. A historical review. *Heart* **76,** 7–12
2. Paul, M., Poyan Mehr, A., and Kreutz, R. (2006) Physiology of local renin-angiotensin systems. *Physiol. Rev.* **86,** 747–803
3. Dinh, D. T., Frauman, A. G., Johnston, C. I., and Fabiani, M. E. (2001) Angiotensin receptors. Distribution, signalling and function. *Clin. Sci.* **100,** 481–492
4. Clarke, N. E., and Turner, A. J. (2012) Angiotensin-converting enzyme 2. The first decade. *Int. J. Hypertens.* **2012,** 307315
5. Santos, R. A., Simoes e Silva, A. C., Maric, C., Silva, D. M., Machado, R. P., de Buhr, I., Heringer-Walther, S., Pinheiro, S. V., Lopes, M. T., Bader, M., Mendes, E. P., Lemos, V. S., Campagnole-Santos, M. J., Schultheiss, H. P., Speth, R., and Walther, T. (2003) Angiotensin-(1–7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc. Natl. Acad. Sci. U.S.A.* **100,** 8258–8263
6. Santos, R. A., Ferreira, A. J., Verano-Braga, T., and Bader, M. (2013) Angiotensin-converting enzyme 2, Angiotensin-(1–7) and Mas. New players of the renin angiotensin system. *J. Endocrinol.* **216,** R1–R17
7. Santos, S. H., Braga, J. F., Mario, E. G., Pôrto, L. C., Rodrigues-Machado Mda, G., Murari, A., Botion, L. M., Alenina, N., Bader, M., and Santos, R. A. (2010) Improved lipid and glucose metabolism in transgenic rats with increased circulating angiotensin-(1–7). *Arterioscler. Thromb. Vasc. Biol.* **30,** 953–961
8. de Kloet, A. D., Krause, E. G., and Woods, S. C. (2010) The renin angiotensin system and the metabolic syndrome. *Physiol. Behav.* **100,** 525–534
9. Kalupahana, N. S., and Moustaid-Moussa, N. (2012) The renin-angiotensin system. A link between obesity, inflammation and insulin resistance. *Obes. Rev.* **13,** 136–149
10. Cassis, L. A., Police, S. B., Yiannikouris, F., and Thatcher, S. E. (2008) Local adipose tissue renin-angiotensin system. *Curr. Hypertens. Rep.* **10,** 93–98
11. Kalupahana, N. S., and Moustaid-Moussa, N. (2012) The adipose tissue renin-angiotensin system and metabolic disorders. A review of molecular mechanisms. *Crit. Rev. Biochem. Mol. Biol.* **47,** 379–390
12. Gupte, M., Boustany-Kari, C. M., Bharadwaj, K., Police, S., Thatcher, S., Gong, M. C., English, V. L., and Cassis, L. A. (2008) ACE2 is expressed in mouse adipocytes and regulated by a high-fat diet. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **295,** R781–R788
13. Engeli, S., Gorzelniak, K., Kreutz, R., Runkel, N., Distler, A., and Sharma, A. M. (1999) Co-expression of renin-angiotensin system genes in human adipose tissue. *J. Hypertens.* **17,** 555–560
14. Goossens, G. H., Blaak, E. E., Arner, P., Saris, W. H., and van Baak, M. A. (2007) Angiotensin II. A hormone that affects lipid metabolism in adipose tissue. *Int. J. Obes.* **31,** 382–384
15. Fujimoto, M., Masuzaki, H., Tanaka, T., Yasue, S., Tomita, T., Okazawa, K., Fujikura, J., Chusho, H., Ebihara, K., Hayashi, T., Hosoda, K., and Nakao, K. (2004) An angiotensin II AT1 receptor antagonist, telmisartan augments glucose uptake and GLUT4 protein expression in 3T3-L1 adipocytes. *FEBS Lett.* **576,** 492–497
16. Oh, Y. B., Kim, J. H., Park, B. M., Park, B. H., and Kim, S. H. (2012) Captopril intake decreases body weight gain via angiotensin-(1–7). *Peptides* **37,** 79–85
17. Liu, C., Lv, X. H., Li, H. X., Cao, X., Zhang, F., Wang, L., Yu, M., and Yang, J. K. (2012) Angiotensin-(1–7) suppresses oxidative stress and improves glucose uptake via Mas receptor in adipocytes. *Acta Diabetol.* **49,** 291–299

18. Kurata, A., Nishizawa, H., Kihara, S., Maeda, N., Sonoda, M., Okada, T., Ohashi, K., Hibuse, T., Fujita, K., Yasui, A., Hiuge, A., Kumada, M., Kuriyama, H., Shimomura, I., and Funahashi, T. (2006) Blockade of angiotensin II type-1 receptor reduces oxidative stress in adipose tissue and ameliorates adipocytokine dysregulation. *Kidney Int.* **70,** 1717–1724
19. Fuentes, P., Acuña, M. J., Cifuentes, M., and Rojas, C. V. (2010) The anti-adipogenic effect of angiotensin II on human preadipose cells involves ERK1,2 activation and PPARG phosphorylation. *J. Endocrinol.* **206,** 75–83
20. Janke, J., Engeli, S., Gorzelniak, K., Luft, F. C., and Sharma, A. M. (2002) Mature adipocytes inhibit *in vitro* differentiation of human preadipocytes via angiotensin type 1 receptors. *Diabetes* **51,** 1699–1707
21. Brücher, R., Cifuentes, M., Acuña, M. J., Albala, C., and Rojas, C. V. (2007) Larger anti-adipogenic effect of angiotensin II on omental preadipose cells of obese humans. *Obesity* **15,** 1643–1646
22. Tomlinson, J. J., Boudreau, A., Wu, D., Abdou Salem, H., Carrigan, A., Gagnon, A., Mears, A. J., Sorisky, A., Atlas, E., and Haché, R. J. (2010) Insulin sensitization of human preadipocytes through glucocorticoid hormone induction of forkhead transcription factors. *Mol. Endocrinol.* **24,** 104–113
23. Hemmrich, K., von Heimburg, D., Cierpka, K., Haydarlioglu, S., and Pallua, N. (2005) Optimization of the differentiation of human preadipocytes *in vitro*. *Differentiation* **73,** 28–35
24. Gregoire, F. M., Smas, C. M., and Sul, H. S. (1998) Understanding adipocyte differentiation. *Physiol. Rev.* **78,** 783–809
25. Ye, F., Zhang, H., Yang, Y. X., Hu, H. D., Sze, S. K., Meng, W., Qian, J., Ren, H., Yang, B. L., Luo, M. Y., Wu, X., Zhu, W., Cai, W. J., and Tong, J. B. (2011) Comparative proteome analysis of 3T3-L1 adipocyte differentiation using iTRAQ-coupled 2D LC-MS/MS. *J. Cell Biochem.* **112,** 3002–3014
26. Than, A., Cheng, Y., Foh, L. C., Leow, M. K., Lim, S. C., Chuah, Y. J., Kang, Y., and Chen, P. (2012) Apelin inhibits adipogenesis and lipolysis through distinct molecular pathways. *Mol. Cell Endocrinol.* **362,** 227–241
27. Hashimoto, T., Igarashi, J., and Kosaka, H. (2009) Sphingosine kinase is induced in mouse 3T3-L1 cells and promotes adipogenesis. *J. Lipid Res.* **50,** 602–610
28. Ramírez-Zacarías, J. L., Castro-Muñozledo, F., and Kuri-Harcuch, W. (1992) Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil red O. *Histochemistry* **97,** 493–497
29. Poulos, S. P., Dodson, M. V., and Hausman, G. J. (2010) Cell line models for differentiation. Preadipocytes and adipocytes. *Exp. Biol. Med.* **235,** 1185–1193
30. Rosen, E. D., Walkey, C. J., Puigserver, P., and Spiegelman, B. M. (2000) Transcriptional regulation of adipogenesis. *Genes Dev.* **14,** 1293–1307
31. Schling, P., Mallow, H., Trindl, A., and Löffler, G. (1999) Evidence for a local renin angiotensin system in primary cultured human preadipocytes. *Int. J. Obes. Relat. Metab. Disord.* **23,** 336–341
32. Lowell, B. B. (1999) PPARγ. An essential regulator of adipogenesis and modulator of fat cell function. *Cell* **99,** 239–242
33. Rosen, E. D., and Spiegelman, B. M. (2000) Molecular regulation of adipogenesis. *Annu. Rev. Cell Dev. Biol.* **16,** 145–171
34. Krämer, B. K., Ritthaler, T., Schweda, F., Ittner, K. P., Scholz, H., Riegger, G. A., and Kurtz, A. (1998) Effects of the angiotensin II type-1 receptor antagonist ZD7155 on angiotensin II-mediated regulation of renin secretion and renal renin gene expression, renal vasoconstriction, and blood pressure in rats. *J. Cardiovasc. Pharmacol.* **31,** 700–705
35. Blankley, C. J., Hodges, J. C., Klutchko, S. R., Himmelsbach, R. J., Chucholowski, A., Connolly, C. J., Neergaard, S. J., Van Nieuwenhze, M. S., Sebastian, A., and Quin, J., 3rd (1991) Synthesis and structure-activity relationships of a novel series of non-peptide angiotensin II receptor binding inhibitors specific for the AT2 subtype. *J. Med. Chem.* **34,** 3248–3260
36. Santos, R. A., Campagnole-Santos, M. J., Baracho, N. C., Fontes, M. A., Silva, L. C., Neves, L. A., Oliveira, D. R., Caligiorne, S. M., Rodrigues, A. R., and Gropen Júnior, C. (1994) Characterization of a new angiotensin antagonist selective for angiotensin-(1–7): evidence that the actions of angiotensin-(1–7) are mediated by specific angiotensin receptors. *Brain Res. Bull.* **35,** 293–298
37. Hutley, L., Shurety, W., Newell, F., McGeary, R., Pelton, N., Grant, J., Herington, A., Cameron, D., Whitehead, J., and Prins, J. (2004) Fibroblast growth factor 1. A key regulator of human adipogenesis. *Diabetes* **53,** 3097–3106
38. Mallow, H., Trindl, A., and Löffler, G. (2000) Production of angiotensin II receptors type one (AT1) and type two (AT2) during the differentiation of 3T3-L1 preadipocytes. *Horm. Metab. Res.* **32,** 500–503
39. Than, A., Tee, W. T., and Chen, P. (2012) Apelin secretion and expression of apelin receptors in 3T3-L1 adipocytes are differentially regulated by angiotensin type 1 and type 2 receptors. *Mol. Cell Endocrinol.* **351,** 296–305
40. Hung, W. W., Hsieh, T. J., Lin, T., Chou, P. C., Hsiao, P. J., Lin, K. D., and Shin, S. J. (2011) Blockade of the renin-angiotensin system ameliorates apelin production in 3T3-L1 adipocytes. *Cardiovasc. Drugs Ther.* **25,** 3–12
41. Rowe, B. P., Saylor, D. L., Speth, R. C., and Absher, D. R. (1995) Angiotensin-(1–7) binding at angiotensin II receptors in the rat brain. *Regul. Pept.* **56,** 139–146
42. Castro, C. H., Santos, R. A., Ferreira, A. J., Bader, M., Alenina, N., and Almeida, A. P. (2005) Evidence for a functional interaction of the angiotensin-(1–7) receptor Mas with AT1 and AT2 receptors in the mouse heart. *Hypertension* **46,** 937–942
43. Tang, Q. Q., Otto, T. C., and Lane, M. D. (2003) Mitotic clonal expansion. A synchronous process required for adipogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **100,** 44–49
44. Qiu, Z., Wei, Y., Chen, N., Jiang, M., Wu, J., and Liao, K. (2001) DNA synthesis and mitotic clonal expansion is not a required step for 3T3-L1 preadipocyte differentiation into adipocytes. *J. Biol. Chem.* **276,** 11988–11995
45. Bost, F., Aouadi, M., Caron, L., and Binétruy, B. (2005) The role of MAPKs in adipocyte differentiation and obesity. *Biochimie* **87,** 51–56
46. Adams, M., Reginato, M. J., Shao, D., Lazar, M. A., and Chatterjee, V. K. (1997) Transcriptional activation by peroxisome proliferator-activated receptor γ is inhibited by phosphorylation at a consensus mitogen-activated protein kinase site. *J. Biol. Chem.* **272,** 5128–5132
47. Xu, J., and Liao, K. (2004) Protein kinase B/AKT 1 plays a pivotal role in insulin-like growth factor-1 receptor signaling induced 3T3-L1 adipocyte differentiation. *J. Biol. Chem.* **279,** 35914–35922
48. Sakaue, H., Ogawa, W., Matsumoto, M., Kuroda, S., Takata, M., Sugimoto, T., Spiegelman, B. M., and Kasuga, M. (1998) Posttranscriptional control of adipocyte differentiation through activation of phosphoinositide 3-kinase. *J. Biol. Chem.* **273,** 28945–28952
49. Giani, J. F., Gironacci, M. M., Muñoz, M. C., Peña, C., Turyn, D., and Dominici, F. P. (2007) Angiotensin-(1–7) stimulates the phosphorylation of JAK2, IRS-1 and Akt in rat heart *in vivo*. Role of the AT1 and Mas receptors. *Am. J. Physiol. Heart Circ. Physiol.* **293,** H1154–H1163
50. Sampaio, W. O., Souza dos Santos, R. A., Faria-Silva, R., da Mata Machado, L. T., Schiffrin, E. L., and Touyz, R. M. (2007) Angiotensin-(1–7) through receptor Mas mediates endothelial nitric oxide synthase activation via Akt-dependent pathways. *Hypertension* **49,** 185–192
51. Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T., and Saltiel, A. R. (1995) PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. *J. Biol. Chem.* **270,** 27489–27494
52. Dang, Z. C., and Lowik, C. W. (2004) Differential effects of PD98059 and U0126 on osteogenesis and adipogenesis. *J. Cell Biochem.* **92,** 525–533
53. Xu, L., Nishimura, K., Jisaka, M., Nagaya, T., and Yokota, K. (2006) Gene expression of arachidonate cyclooxygenase pathway leading to the delayed synthesis of prostaglandins E2 and F2α in response to phorbol 12-myristate 13-acetate and action of these prostanoids during life cycle of adipocytes. *Biochim. Biophys. Acta.* **1761,** 434–444
54. Rhee, S. D., Sung, Y. Y., Jung, W. H., and Cheon, H. G. (2008) Leptin inhibits rosiglitazone-induced adipogenesis in murine primary adipocytes. *Mol. Cell Endocrinol.* **294,** 61–69
55. Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, R. F. (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem.* **269,** 5241–5248
56. Frühbeck, G., Gómez-Ambrosi, J., Muruzábal, F. J., and Burrell, M. A. (2001) The adipocyte. A model for integration of endocrine and metabolic signaling in energy metabolism regulation. *Am. J. Physiol. Endocrinol. Metab.* **280,** E827–E847

57. Schling, P., and Löffler, G. (2002) Cross talk between adipose tissue cells. Impact on pathophysiology. *News Physiol. Sci.* **17,** 99–104
58. Attie, A. D., and Scherer, P. E. (2009) Adipocyte metabolism and obesity. *J. Lipid Res.* **50,** S395–S399
59. Than, A., Ye, F., Xue, R., Ong, J. W., Poh, C. L., and Chen, P. (2011) The crosstalks between adipokines and catecholamines. *Mol. Cell Endocrinol.* **332,** 261–270
60. Peckett, A. J., Wright, D. C., and Riddell, M. C. (2011) The effects of glucocorticoids on adipose tissue lipid metabolism. *Metabolism* **60,** 1500–1510
61. Guerre-Millo, M. (2004) Adipose tissue and adipokines. For better or worse. *Diabetes Metab.* **30,** 13–19
62. Karastergiou, K., and Mohamed-Ali, V. (2010) The autocrine and paracrine roles of adipokines. *Mol. Cell Endocrinol.* **318,** 69–78
63. Ye, F., Than, A., Zhao, Y., Goh, K. H., and Chen, P. (2010) Vesicular storage, vesicle trafficking, and secretion of leptin and resistin. The similarities, differences, and interplays. *J. Endocrinol.* **206,** 27–36
64. Fu, Y., Luo, N., Klein, R. L., and Garvey, W. T. (2005) Adiponectin promotes adipocyte differentiation, insulin sensitivity, and lipid accumulation. *J. Lipid Res.* **46,** 1369–1379
65. Qiao, L., Kinney, B., Schaack, J., and Shao, J. (2011) Adiponectin inhibits lipolysis in mouse adipocytes. *Diabetes* **60,** 1519–1527
66. Frühbeck, G., Aguado, M., and Martínez, J. A. (1997) *In vitro* lipolytic effect of leptin on mouse adipocytes. Evidence for a possible autocrine/paracrine role of leptin. *Biochem. Biophys. Res. Commun.* **240,** 590–594
67. Zhu, S., Sun, F., Li, W., Cao, Y., Wang, C., Wang, Y., Liang, D., Zhang, R., Zhang, S., Wang, H., and Cao, F. (2011) Apelin stimulates glucose uptake through the PI3K/Akt pathway and improves insulin resistance in 3T3-L1 adipocytes. *Mol. Cell Biochem.* **353,** 305–313
68. Danforth, E., Jr. (2000) Failure of adipocyte differentiation causes type II diabetes mellitus? *Nat. Genet.* **26,** 13
69. Freedland, E. S. (2004) Role of a critical visceral adipose tissue threshold (CVATT) in metabolic syndrome. Implications for controlling dietary carbohydrates. A review. *Nutr. Metab.* **1,** 12
70. Jernås, M., Palming, J., Sjöholm, K., Jennische, E., Svensson, P. A., Gabrielsson, B. G., Levin, M., Sjögren, A., Rudemo, M., Lystig, T. C., Carlsson, B., Carlsson, L. M., and Lönn, M. (2006) Separation of human adipocytes by size. Hypertrophic fat cells display distinct gene expression. *FASEB J.* **20,** 1540–1542
71. Kubota, N., Terauchi, Y., Miki, H., Tamemoto, H., Yamauchi, T., Komeda, K., Satoh, S., Nakano, R., Ishii, C., Sugiyama, T., Eto, K., Tsubamoto, Y., Okuno, A., Murakami, K., Sekihara, H., Hasegawa, G., Naito, M., Toyoshima, Y., Tanaka, S., Shiota, K., Kitamura, T., Fujita, T., Ezaki, O., Aizawa, S., and Kadowaki, T. (1999) PPARγ mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. *Mol. Cell* **4,** 597–609
72. Lebovitz, H. E., and Banerji, M. A. (2005) Point. Visceral adiposity is causally related to insulin resistance. *Diabetes Care* **28,** 2322–2325
73. Miller, M., Stone, N. J., Ballantyne, C., Bittner, V., Criqui, M. H., Ginsberg, H. N., Goldberg, A. C., Howard, W. J., Jacobson, M. S., Kris-Etherton, P. M., Lennie, T. A., Levi, M., Mazzone, T., and Pennathur, S. (2011) Triglycerides and cardiovascular disease. A scientific statement from the American Heart Association. *Circulation* **123,** 2292–2333
74. Ibrahim, M. M. (2010) Subcutaneous and visceral adipose tissue. Structural and functional differences. *Obes. Rev.* **11,** 11–18
75. Adams, M., Montague, C. T., Prins, J. B., Holder, J. C., Smith, S. A., Sanders, L., Digby, J. E., Sewter, C. P., Lazar, M. A., Chatterjee, V. K., and O'Rahilly, S. (1997) Activators of peroxisome proliferator-activated receptor γ have depot-specific effects on human preadipocyte differentiation. *J. Clin. Invest.* **100,** 3149–3153
76. Laplante, M., Festuccia, W.T., Soucy, G., Gélinas, Y., Lalonde, J., Berger, J. P., and Deshaies, Y. (2006) Mechanisms of the depot specificity of peroxisome proliferator-activated receptor γ action on adipose tissue metabolism. *Diabetes* **55,** 2771–2778
77. Giani, J. F., Mayer, M. A., Muñoz, M. C., Silberman, E. A., Höcht, C., Taira, C. A., Gironacci, M. M., Turyn, D., and Dominici, F. P. (2009) Chronic infusion of angiotensin-(1–7) improves insulin resistance and hypertension induced by a high-fructose diet in rats. *Am. J. Physiol. Endocrinol. Metab.* **296,** E262–271
78. Santos, S. H., Fernandes, L. R., Mario, E. G., Ferreira, A. V., Pôrto, L. C., Alvarez-Leite, J. I., Botion, L. M., Bader, M., Alenina, N., and Santos, R. A. (2008) Mas deficiency in FVB/N mice produces marked changes in lipid and glycemic metabolism. *Diabetes* **57,** 340–347
79. Sarzani, R., Marcucci, P., Salvi, F., Bordicchia, M., Espinosa, E., Mucci, L., Lorenzetti, B., Minardi, D., Muzzonigro, G., Dessi-Fulgheri, P., and Rappelli, A. (2008) Angiotensin II stimulates and atrial natriuretic peptide inhibits human visceral adipocyte growth. *Int. J. Obes.* **32,** 259–267
80. Olivares-Reyes, J. A., Arellano-Plancarte, A., and Castillo-Hernandez, J. R. (2009) Angiotensin II and the development of insulin resistance. Implications for diabetes. *Mol. Cell Endocrinol.* **302,** 128–139
81. He, H., Yang, D., Ma, L., Luo, Z., Ma, S., Feng, X., Cao, T., Yan, Z., Liu, D., Tepel, M., and Zhu, Z. (2010) Telmisartan prevents weight gain and obesity through activation of peroxisome proliferator-activated receptor-δ-dependent pathways. *Hypertension* **55,** 869–879
82. Kakuma, T., Gotoh, K., Masaki, T., Itateyama, E., Abe, N., and Yoshimatsu, H. (2010) Telmisartan reduced abdominal circumference and body weight with decreasing triglyceride level in patients with type 2 diabetes and metabolic syndrome. *Obes. Res. Clin. Pract.* **4,** e145–e152
83. Henriksen, E. J., Jacob, S., Kinnick, T. R., Teachey, M. K., and Krekler, M. (2001) Selective angiotensin II receptor antagonism reduces insulin resistance in obese Zucker rats. *Hypertension* **38,** 884–890
84. Engeli, S., Negrel, R., and Sharma, A. M. (2000) Physiology and pathophysiology of the adipose tissue renin-angiotensin system. *Hypertension* **35,** 1270–1277
85. Thatcher, S., Yiannikouris, F., Gupte, M., and Cassis, L. (2009) The adipose renin-angiotensin system. Role in cardiovascular disease. *Mol. Cell Endocrinol.* **302,** 111–117
86. Heffelfinger, S. C. (2007) The renin angiotensin system in the regulation of angiogenesis. *Curr. Pharm. Des.* **13,** 1215–1229
87. Ramracheya, R. D., Muller, D. S., Wu, Y., Whitehouse, B. J., Huang, G. C., Amiel, S. A., Karalliedde, J., Viberti, G., Jones, P. M., and Persaud, S. J. (2006) Direct regulation of insulin secretion by angiotensin II in human islets of Langerhans. *Diabetologia* **49,** 321–331