SLC3A2 and SLC7A2 Mediate the Exogenous Putrescine-Induced Adipocyte Differentiation

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Exogenous polyamines are able to induce life span and improve glucose homeostasis and insulin sensitivity. However, the effects of exogenous polyamines on adipocyte differentiation and which polyamine transporters mediate them have not been elucidated yet. Here, we identified for the first time that exogenous polyamines can clearly stimulate adipocyte differentiation through polyamine transporters, solute carrier family 3 member A2 (SLC3A2) and SLC7A1. Exogenous polyamines markedly promote 3T3-L1 adipocyte differentiation by increasing the intracellular lipid accumulation and the expression of both adipogenic and lipogenic genes in a concentration-dependent manner. In particular, exogenous putrescine mainly regulates adipocyte differentiation in the early and intermediate stages. Moreover, we have assessed the expression of polyamine transporter genes in 3T3-L1 preadipocytes and adipocytes. Interestingly, the putrescine-induced adipocyte differentiation was found to be significantly suppressed in response to a treatment with a polyamine transporter inhibitor (AMXT-1501). Furthermore, knockdown experiments using siRNA that specifically targeted SLC3A2 or SLC7A2, revealed that both SLC3A2 and SLC7A2 act as important transporters in the cellular importing of exogenous putrescine. Thus, the exogenous putrescine entering the adipocytes via cellular transporters is involved in adipogenesis through a modulation of both the mitotic clonal expansion and the expression of master transcription factors. Taken together, these results suggest that exogenous polyamines (such as putrescine) entering the adipocytes through polyamine transporters, can stimulate adipogenesis.

Keywords: adipocyte, adipogenesis, differentiation, polyamine, putrescine

INTRODUCTION

Polyamines, including putrescine, spermidine, and spermine, are ubiquitous polycations, which are present in the mammalian cells in millimolar concentrations (Casero and Pegg, 2009; Pegg and Casero, 2011). The polyamines can readily bind to negatively charged biomolecules including DNA, RNA, proteins, and phospholipids (Li et al., 2020). Therefore, they play crucial roles in fundamental cellular processes such as cell growth, proliferation, migration, and differentiation (Casero et al., 2018; Igarashi and Kashiwagi, 2010; Li et al., 2020; Pegg and Casero, 2011). Intracellular polyamine concentrations are tightly regulated by a complex regulatory mechanism involving *de novo* synthesis, catabolism, and transport across the plasma membrane (Ramos-Molina et al., 2019). When polyamine metabolism is pharmacologically or genetically altered, many relevant biochemical, and cellular processes are affected (Brenner et al., 2015; Janne et al., 2004; Landau et al., 2012; Ramos-Molina et al., 2019).

In cancer, polyamine metabolism is frequently dysregulated, indicating that elevated polyamine levels are necessary for cell proliferation and tumorigenesis (Casero et al., 2018; Murray-Stewart et al., 2016). Moreover, several studies have shown that increased levels of polyamines in white adipose tissue, liver, or skeletal muscle could stimulate energy expenditure and confer resistance to obesity and non-alcoholic fatty liver diseases (Bonhoure et al., 2015; Kraus et al., 2014; Ramos-Molina et al., 2019). Alterations in polyamine levels also affect pancreatic β-cell functions including impaired glucose-stimulated insulin secretion (Cerrada-Gimenez et al., 2012).

Adipocytes are not only the major sites of energy storage in the body, but also have critical endocrine functions in the regulation of energy metabolism (Ali et al., 2013; Song and Deng, 2020). Adipocyte differentiation is a complex process accompanied by coordinated changes in the adipocytes' morphology, hormone sensitivity, and gene expression (Kim et al., 2021; Lefterova and Lazar, 2009; Linhart et al., 2001; Rosen et al., 2000; Tontonoz et al., 1994). It has been shown that the intracellular polyamine metabolism is involved in adipogenesis via the inhibition of ornithine decarboxylase (ODC; the rate-limiting enzyme in the polyamine biosynthesis) with α-difluoromethylornithine (DFMO) (Ishii et al., 2012). In addition, it has been shown that a polyamine depletion can reduce adipogenesis through the inhibition of both the mitotic clonal expansion (MCE) and the expression of peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/ enhancer-binding protein alpha (C/EBPα) (Brenner et al., 2015; Tanaka et al., 1997). However, the effects of exogenous polyamines on adipocyte differentiation have not been elucidated yet.

In the present study, we have investigated the activating effect of an exogenous polyamine (putrescine) on adipogenesis, and we have identified for the first time that the polyamine transporters SLC3A2 and SLC7A2 as responsible for the entry of putrescine into the adipocytes. We, herein, also show that a treatment with exogenous putrescine can reduce the C/EBP homologous protein (CHOP) expression, which is correlated with the induction of CCAAT/enhancer-binding protein beta (C/EBPβ) expression. The latter results into the activation of MCE and an increment in the expression of PPARγ and C/EBPα, thereby leading to a stimulation of adipocyte differentiation.

MATERIALS AND METHODS

Cell culturing
3T3-L1 cells were purchased from the American Type Culture Collection (ATCC, USA). 3T3-L1 preadipocytes were maintained in Dulbecco's modified Essential medium (DMEM; Welgene, Korea) supplemented with 10% bovine calf serum (Welgene), 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37℃ with 10% CO_2. The differentiation of 3T3-L1 cells was induced by an induction medium containing 0.2 × MDI in order to examine the stimulating effect of the polyamine, as described previously (Yeon et al., 2021). In particular, in order to induce differentiation, 3T3-L1 preadipocytes were seeded in a 12-well culture plate at a density of 5.0×10^4 cells per

well. Two days after achieving confluence (D0), the adipocyte differentiation was initiated by culturing the growth-arrested 3T3-L1 preadipocytes for 48 h with a differentiation medium containing DMEM that was supplemented with 10% fetal bovine serum (FBS; Welgene) as well as with a cocktail of hormones (0.2 × MDI), 0.1 mM 3-isobutyl-1-methylxanthine (M; Sigma Aldrich, USA), 0.2 μM dexamethasone (D; Sigma Aldrich), and 0.2 μg/ml insulin (I; Sigma Aldrich). Starting from day 2 (D2), the cells were cultured in an adipocyte maintenance medium containing DMEM that was supplemented with 10% FBS as well as with 1 μg/ml insulin; the medium was renewed every 48 h until day 6 (D6).

Animals
Male C57BL/6J mice (8 to 12 weeks old) were purchased from G-Bio (Korea). The mice were housed in a standard environment with controlled temperature (23°C ± 2°C) and relative humidity (55% ± 10%), and a 12-h light/dark cycle. Animals were freely fed standard chow diets (PicoLab Rodent Diet 5053; Purina, USA) and had access to ion-sterilized tap water. Ethical approval was obtained from the Institutional Animal Care and Use Committee (IACUC) of Mokpo National University (Korea) (approval No. MNU-IACUC-2021-020). All animal experiments were performed in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals as well as the IACUC guidelines.

Isolation of stromal vascular cells (SVCs), and primary preadipocyte differentiation
Primary SVCs (primary preadipocytes) were obtained from the subcutaneous fat of male C57BL/6J mice. Briefly, subcutaneous fat pads were minced in a collagenase buffer, and were digested at 37°C with constant agitation at 160 rpm for 30 min. Digestion was stopped by adding 10 ml of serum-containing medium. After filtration through a 100-μm cell strainer, the solution was centrifuged for 5 min at 380 g, the supernatant was removed, and the pellet was resuspended and incubated with red blood cell lysis buffer (Thermo Fisher Scientific, USA). Again, cells were filtered through a 40-μm cell strainer, and were seeded after centrifugation. For adipocyte differentiation, the primary preadipocytes were first cultured to confluence in a DMEM/F12 medium containing 10% FBS, penicillin/streptomycin (PS), and glutamine, and were subsequently induced with a differentiation cocktail consisting of 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, 1 μg/ml insulin, 0.2 mM indomethacin, and 1 μM rosiglitazone in DMEM supplemented with 10% FBS, PS, and glutamine. After 2 days, the medium was replaced by a DMEM containing 10% FBS, PS, glutamine, 1 μg/ml insulin, and 1 μM rosiglitazone; thereafter, medium renewal took place every other day, as described previously (El Ouarrat et al., 2020).

Cell viability assay
Cell viability was quantified by using the WST-8 Cell Viability Assay Kit (BIOMAX, Korea) according to the manufacturer's protocol. This method is based on the ability of viable cells to metabolize the tetrazolium salt WST-8 into formazan through their mitochondrial dehydrogenases. 3T3-L1 preadipocytes

were equally seeded at a density of 1×10^4 cells per well in 96-well plates, and were cultured for 24 h in the culture medium. Subsequently, the cells were treated with various concentrations of putrescine for 24, 48, and 72 h prior to the addition of the WST-8 reagent. After an incubation at 37℃ for 1 h, the samples were quantified spectrophotometrically by measuring the absorbance of the formazan product at 450 nm with the use of an iMark™ microplate reader (Bio-Rad Laboratories, USA). The percentage of viable cells was calculated by defining the cell viability without treatment as 100%.

Oil red O staining

The differentiated 3T3-L1 cells were carefully washed twice with phosphate-buffered saline (PBS), and were then fixed with 3.7% formalin for 10 min. After removing formalin, 60% isopropanol was added to each well for 3 min. Subsequently, the cells were then incubated with an oil red O solution for 20 min, and were washed three times with distilled water. The staining of lipid droplets and the cell morphology was ascertained microscopically by using a microscope (NIB410; Nexcope, USA) at a ×200 magnification. The lipid droplets were quantified by dissolving oil red O in 100% isopropanol, and by measuring the optical density at 500 nm with the use of an iMark™ microplate reader (Bio-Rad Laboratories).

Quantitative real-time PCR

Total RNA was extracted with the use of the RiboEx™ reagent (GeneAll Biotechnology, Korea). The synthesis of cDNA was performed by using the ReverTra Ace™ qPCR RT kit (Toyobo, Japan). Quantitative real-time PCR was performed by using a CFX Connect Real-Time PCR system (Bio-Rad Laboratories) and SYBRGreen PCR Master Mix (Bio-Rad Laboratories). 36B4 was used as an internal control in order to calculate the expression levels. The sequences of the primers used in the experiment are the same as in a previous study (Yeon et al., 2021).

Western blotting analysis

Cells were lysed with the use of the Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) containing an Xpert protease inhibitor cocktail (GenDEPOT, USA), and were then centrifuged at 12,000 rpm for 10 min at 4°C. The supernatants were separated by precast gels (Bio-Rad Laboratories) using a running buffer, and were subsequently electrotransferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked for 2 h in Tris-buffered saline with Tween 20 (TBST; containing 20 mM Tris-HCl, 150 mM NaCl, 0.2% Tween 20, pH 7.4) supplemented with 5% bovine serum albumin, and were then incubated with an anti-PPARγ antibody (Cat. No. 2443; Cell Signaling Technology, USA), an anti-adiponectin antibody (Cat. No. MA1-054; Thermo Fisher Scientific), and an anti-HSP90 antibody (Cat. No. SC-13119; Santa Cruz Biotechnology, USA), at 4°C overnight. After washing with fresh TBST, the membrane was incubated with secondary antibodies conjugated with horseradish peroxidase specific to rabbit or mouse IgG (1:5,000 dilution; Bio-Rad Laboratories), and were visualized by using the ECL system (Bio-Rad Laboratories) followed by an iBright CL1500 imaging system (Thermo Fisher Scientific).

Transfection of siRNA

siRNAs targeting polyamine transporters were purchased from Sigma-Aldrich (USA). 3T3-L1 preadipocytes were seeded in a 12-well culture plate at a density of 5.0×10^4 cells per well. The next day, the medium was replaced with an Opti-MEM, 30 min prior to transfection. Subsequently, by using Lipofectamine™ 3000 reagent (Invitrogen, USA), the siRNA transfection was carried out in accordance to the manufacturer's experimental procedure.

Cell counting with nuclear staining

Cells were washed twice with Dulbecco's PBS (DPBS), and were fixed with 3.7% formaldehyde, at room temperature, for 30 min. Subsequently, the cells were washed twice with DPBS, and were stained with 1 μg/ml Hoechst 33342 (Invitrogen) for 10 min. Finally, after washing with DPBS, the staining of the nucleus and the cell morphology were ascertained microscopically by using a microscope (NIB410; Nexcope) at a ×200 magnification. The number of cells was measured by using ImageJ.

Statistical analysis

The results are shown as mean ± SEM, while n denotes the number of wells analyzed. Statistical analyses were performed by using Prism 9 (GraphPad Software, USA). Data were analyzed via two-way ANOVA followed by Tukey's post-hoc test at $\alpha = 0.05$. A P value lower than <0.05 was considered as significant.

RESULTS

Exogenous putrescine induces adipocyte differentiation

Firstly, in order to examine the cytotoxic effect of exogenous putrescine on 3T3-L1 preadipocytes, we performed a cell viability assay (WST-8). As shown in Fig. 1A, cells exposed to 0.1-10 mM putrescine for 24, 48, and 72 h did not exhibit any cytotoxicity. Secondly, in order to investigate the effects of exogenous putrescine on the 3T3-L1 adipocyte differentiation, post-confluent 3T3-L1 preadipocytes were treated with various concentrations of putrescine during adipogenesis. Subsequently, the intracellular lipid accumulation was measured through an oil red O staining assay, thereby revealing that exogenous putrescine can significantly increase lipid accumulation in a concentration-dependent manner (Figs. 1B and 1C).

In order to test the effects of exogenous putrescine on the expression of genes related to adipocyte differentiation, the levels of each mRNA were measured through quantitative real-time PCR. We observed significantly increased mRNA levels of adipogenic genes (such as PPARγ, C/EBPα, C/EBPβ, fatty acid binding protein 4 [FABP4], and adiponectin) in putrescine-treated cells (Fig. 2A). Interestingly, the expression levels of adipogenic genes were found to be increased in a manner proportional to that of the putrescine concentrations used, while the expression levels of the preadipocyte factor-1 (Pref-1; a preadipocyte marker gene), were decreased. In addition,
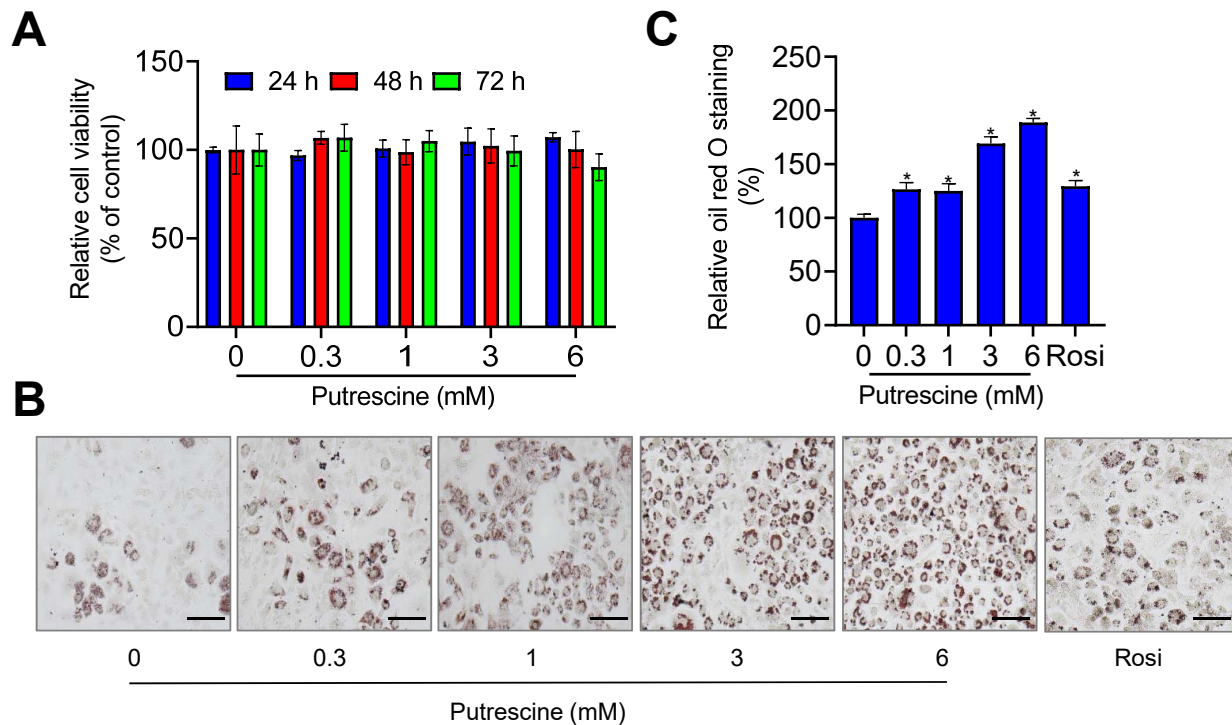
Fig. 1. The effects of putrescine on 3T3-L1 adipocyte differentiation. (A) 3T3-L1 preadipocytes were cultured with various concentrations of putrescine for 24, 48, and 72 h, and cell viability was analyzed by using a WST-8 based colorimetric assay kit. The values are expressed relatively to the control group. All data are presented as the mean ± SEM of two independent experiments (n = 5) for each condition. (B) Cells were differentiated for 6 days in the presence of exogenous putrescine, as indicated. After differentiation, the cells were stained with oil red O. Scale bars = 100 μm. (C) The results are presented as mean ± SEM of the oil red O absorbance values measured at 490 nm. *$P < 0.05$ versus the adipocyte group (two-way ANOVA followed by Tukey's post hoc test).

the protein levels of PPARγ and adiponectin were found to be upregulated in a putrescine concentration-dependent manner (Fig. 2B).

Subsequently and in order to investigate the effect of exogenous putrescine on lipogenesis, we performed quantitative real-time PCR so as to measure the expression levels of lipogenic genes such as those of sterol regulatory element binding protein 1c (SREBP1c), stearoyl-CoA desaturase (SCD1), SCD2, fatty acid synthase (FAS), and acetyl-CoA carboxylase (ACC). Based on our results, exogenous putrescine markedly stimulated the expression of the aforementioned genes at the transcriptional level (Fig. 2C). Surprisingly, a treatment with 6 mM of putrescine exerted a stronger stimulating effect on the mRNA expression of these lipogenesis marker genes than the positive control group (10 μM rosiglitazone).

Additionally, whether other polyamines can stimulate adipocyte differentiation, we examined the effects of spermidine and spermine on adipogenesis (Supplementary Fig. S1). Although the concentrations are different, both spermidine (50 μM) and spermine (10 μM) also promoted 3T3-L1 adipocyte differentiation.

Exogenous putrescine acts on adipocyte differentiation at the early and intermediate stages of differentiation
In order to verify the critical stage of adipocyte differentiation

affected by exogenous putrescine, post-confluent 3T3-L1 preadipocytes were treated with 3 mM putrescine at different timepoints, as illustrated in Fig. 3A. As shown in Figs. 3B-3F, the treatment groups of putrescine on days 0-2 (treatment #3) and days 0-4 (treatment #6) resulted in a marked stimulating activity, similar to the adipogenic effect of the putrescine treatment group on days 0-6 (treatment #2). In the two groups (treatments #3 and #6), the mRNA levels of the adipogenic and lipogenic genes were significantly upregulated in response to the putrescine treatment (Figs. 3D-3F). Moreover, differentiating 3T3-L1 preadipocytes that were treated with putrescine on days 2-4 (treatment #4) and days 2-6 (treatment #7), exhibited a weaker activation of adipocyte differentiation than treatments #2, #3, and #6. On the other hand, when putrescine was administered only during the late stage of the adipocyte differentiation (days 4-6; treatment #5), we observed no promoting activity at all. These results suggest that the adipogenic effect of exogenously-administered putrescine was due to the regulation of intracellular events taking place during the early and intermediate stages of adipogenesis.

Exogenous putrescine also induces adipocyte differentiation of primary SVCs
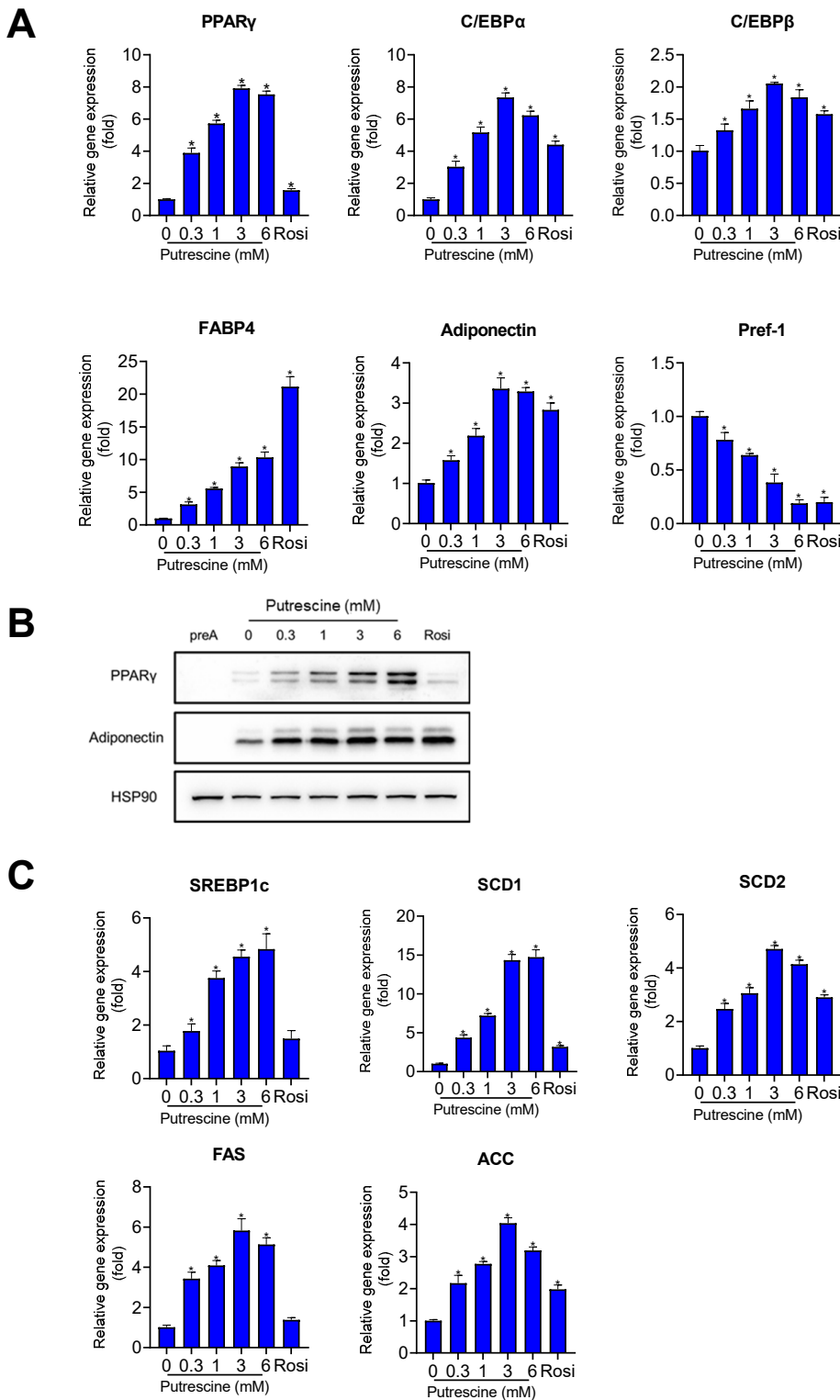Subsequently and in order to elucidate the adipogenic effect

Fig. 2. The effects of putrescine on 3T3-L1 adipocyte differentiation. (A-C) Cells were differentiated into adipocytes for 6 days by using different induction media containing 0.2 × MDI. (A) The mRNA expression of adipogenic genes, as analyzed through quantitative real-time PCR. (B) PPARγ and adiponectin expression, as determined by Western blotting. (C) mRNA expression of lipogenic genes, as analyzed through quantitative real-time PCR. All data are presented as the mean ± SEM of two independent experiments (n = 4). *$P < 0.05$ versus the adipocyte group (two-way ANOVA followed by Tukey's post hoc test). PPARγ, peroxisome proliferator-activated receptor gamma; C/EBPα, CCAAT/enhancer-binding protein alpha; C/EBPβ, CCAAT/enhancer-binding protein beta; FABP4, fatty acid binding protein 4; Pref-1, preadipocyte factor-1; SREBP1c, sterol regulatory element binding protein 1c; SCD1, stearoyl-CoA desaturase 1; SCD2, stearoyl-CoA desaturase 2; FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase.

of exogenous putrescine on 3T3-L1 adipocyte differentiation, we examined primary SVCs. As shown Fig. 4, comparable results were observed in primary SVCs. The mRNA levels of adipocyte-specific genes (such as PPARγ, C/EBPα, C/EBPβ, FABP4, and adiponectin) were found to be stimulated by

exogenous putrescine. The protein levels of PPARγ and adiponectin were also induced by putrescine in SVCs undergoing adipogenesis. In contrast, the mRNA level of the Pref-1 gene was significantly reduced by putrescine treatment. In addition, the mRNA levels of lipogenic genes, including those
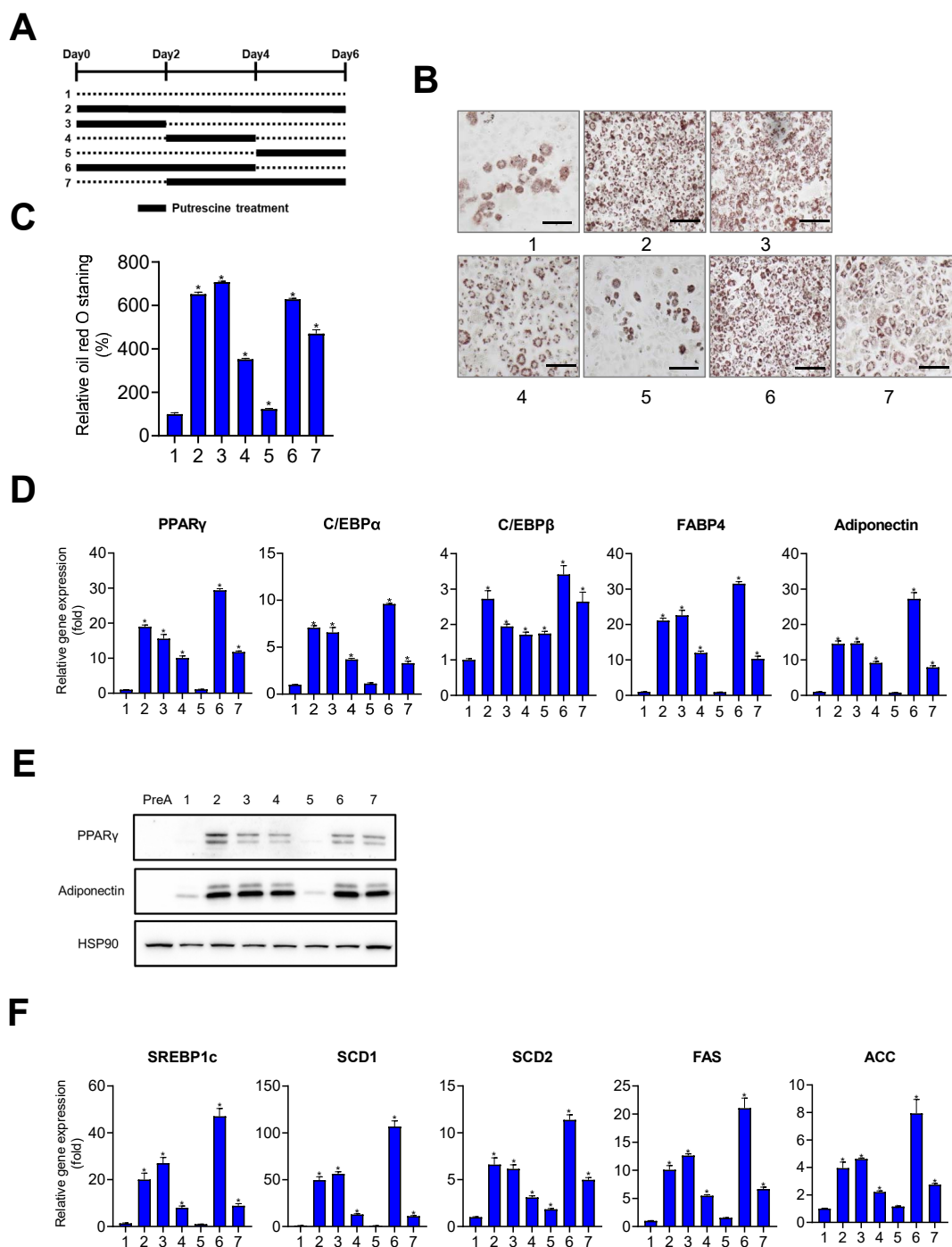
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Fig. 3. Stage-specific effect of putrescine on adipocyte differentiation. (A) Schematic representation of the experimental scheme for adipogenesis via a treatment with 6 mM of putrescine for different periods. (B and C) After differentiation, the cells were stained with oil red O in order to quantify the triglyceride accumulation. Scale bars = 100 μm. The results are presented as mean ± SEM of the oil red O absorbance values measured at 490 nm. (D and F) After adipocyte differentiation, the mRNA expression of adipogenic and lipogenic genes was analyzed through quantitative real-time PCR. All data are presented as the mean ± SEM of two independent experiments (n = 4). *P < 0.05 versus the adipocyte group (two-way ANOVA followed by Tukey's post hoc test). (E) Western blotting was also performed. PPARγ, peroxisome proliferator-activated receptor gamma; C/EBPα, CCAAT/enhancer-binding protein alpha; C/EBPβ, CCAAT/enhancer-binding protein beta; FABP4, fatty acid binding protein 4; SREBP1c, sterol regulatory element binding protein 1c; SCD1, stearoyl-CoA desaturase 1; SCD2, stearoyl-CoA desaturase 2; FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase.
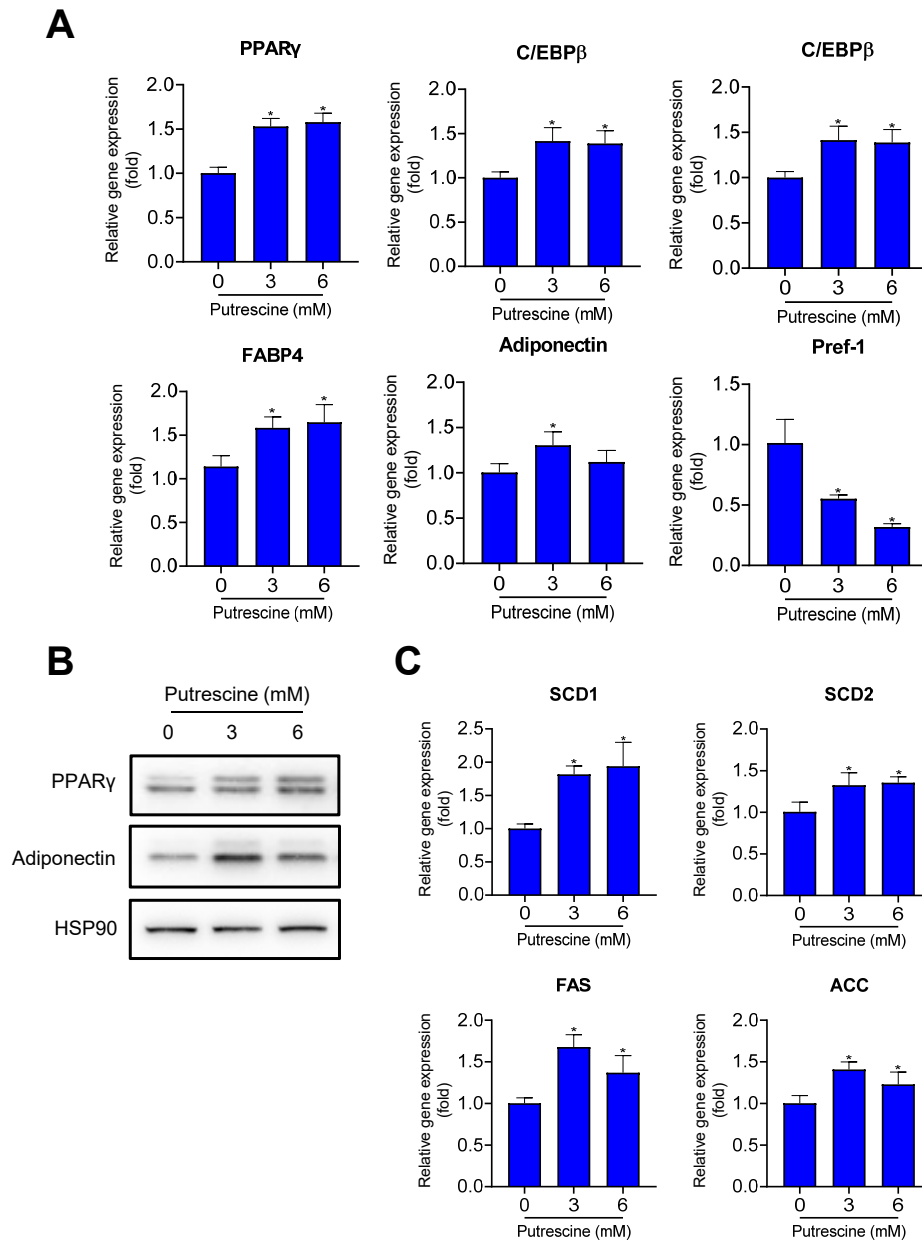
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Fig. 4. The effects of putrescine on adipocyte differentiation of primary stromal vascular cells (SVCs). Cells were differentiated into mature adipocytes for 6 days in the absence (control) or presence of various concentrations of putrescine. (A and C) The presence of polyamines was determined, and the mRNA expression of adipogenic and lipogenic genes was analyzed by quantitative real-time PCR. All data are presented as the mean ± SEM of two independent experiments (n = 4). $*P < 0.05$ versus the adipocyte group (two-way ANOVA followed by Tukey's post hoc test). (B) PPARγ and adiponectin expressions were determined by immunoblotting. PPARγ, peroxisome proliferator-activated receptor gamma; C/EBPα, CCAAT/enhancer-binding protein alpha; C/EBPβ, CCAAT/enhancer-binding protein beta; FABP4, fatty acid binding protein 4; Pref-1, preadipocyte factor-1; SCD1, stearoyl-CoA desaturase 1; SCD2, stearoyl-CoA desaturase 2; FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase.

of SCD1, SCD2, FAS, and ACC, were also found to be stimulated. These findings suggest that the stimulating effect of putrescine on adipocyte differentiation is promising.

Polyamine transporter inhibitor (AMXT-1501) significantly decreases the putrescine-induced adipogenesis

In order to investigate whether the polyamine transporter is involved in the entry of putrescine into the cells, we assessed the expression of polyamine transporter genes by quantitative real-time PCR. As shown in Fig. 5A, the mRNA levels of polyamine transporters, including those of glypican 1 (GPC1), the solute carrier family 3 member A2 (SLC3A2), SLC7A1, SLC7A2, SLC18B1, and SLC22A8, were detected in both preadipocytes and adipocytes. Intriguingly, the expression of SLC18B1 in adipocytes was higher than that observed in preadipocytes. Subsequently and in order to examine whether the inhibition of the polyamine transporter activity can block the adipogenic effect of exogenous putrescine, we employed AMXT-1501; a novel polyamine transporter inhibitor (Samal et al., 2013). As expected, the inhibition of the polyamine transporter with AMXT-1501 significantly reduced the putrescine-induced adipocyte differentiation (Figs. 5B-5D). AMXT-1501 clearly decreased the expression of the putrescine-induced adipogenic genes, including those of PPARγ, C/EBPα, C/EBPβ, FABP4, and adiponectin. Similarly, AMXT-1501 also inhibited the expression of the putrescine-induced lipogenic genes (including SREBP1c, SCD1, SCD2, FAS, and ACC). These data imply that polyamine transporters might mediate the cellular entry of exogenous putrescine, thereby promoting adipocyte differentiation.

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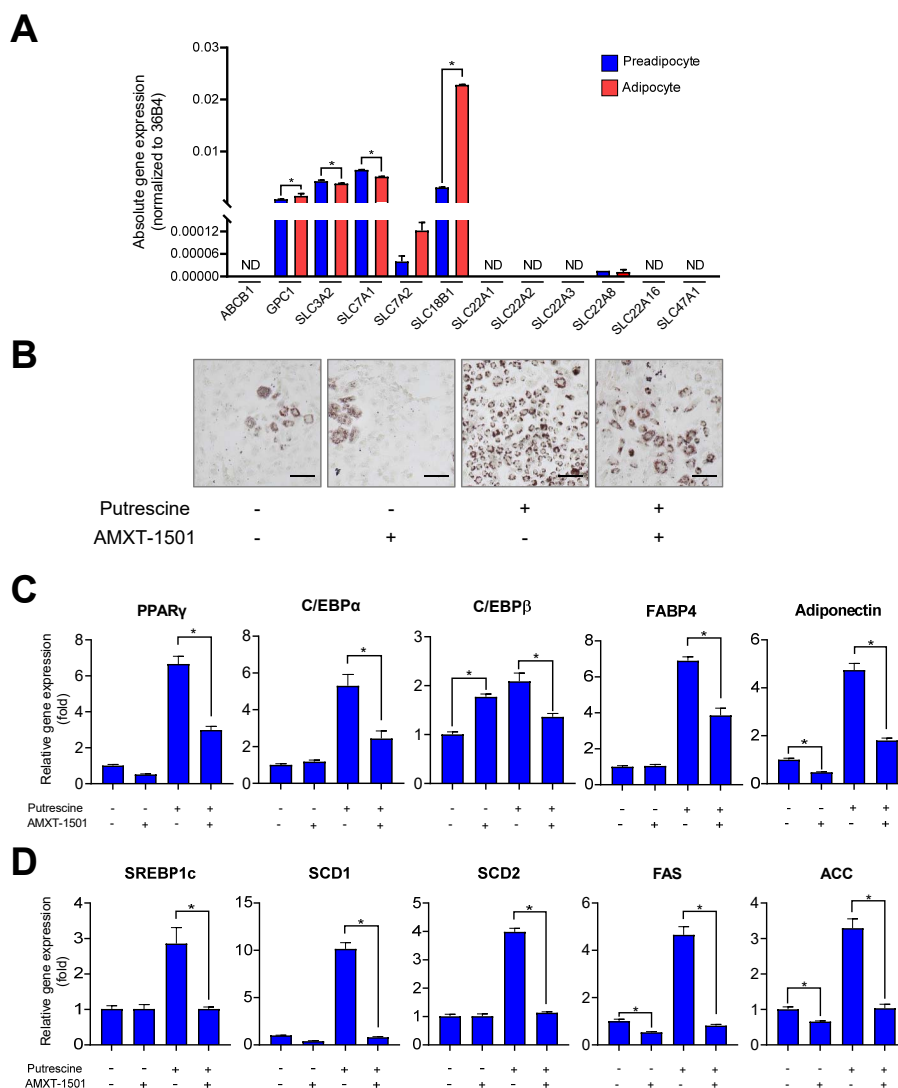|			
Putrescine	−	−	+	+
AMXT-1501	−	+	−	+

C

D

Fig. 5. The expression of polyamine transporters in 3T3-L1 preadipocytes and adipocytes. (A) Analysis of the mRNA expression of polyamine transporter genes in 3T3-L1 preadipocytes and adipocytes. (B-D) During the differentiation of 3T3-L1, cells were exposed to 3 mM putrescine for 2 days with or without 1 h pretreatment of a polyamine transporter inhibitor AMXT-1501, as indicated. (B) Cells were stained with oil red O in order to check the triglyceride accumulation. Scale bars = 100 μm. (C and D) After the adipocyte differentiation, the mRNA expressions of the PPARγ, C/EBPα, C/EBPβ, FABP4, adiponectin, SREBP1c, SCD1, SCD2, FAS, and ACC genes were analyzed by quantitative real-time PCR. All data are presented as mean ± SEM. *$P < 0.05$ versus the control group (two-way ANOVA followed by Tukey's post hoc test). ND, non-detected; PPARγ, peroxisome proliferator-activated receptor gamma; C/EBPα, CCAAT/enhancer-binding protein alpha; C/EBPβ, CCAAT/enhancer-binding protein beta; FABP4, fatty acid binding protein 4; SREBP1c, sterol regulatory element binding protein 1c; SCD1, stearoyl-CoA desaturase 1; SCD2, stearoyl-CoA desaturase 2; FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase.

The knockdown of polyamine transporters SLC3A2 and SLC7A2 attenuates the putrescine-induced adipogenesis

In order to identify which polyamine transporter(s) can mediate the entering of putrescine into the cells, we conducted knockdown experiments with the use of siRNAs. After the transfection of each siRNA of the polyamine transporters, the transfected 3T3-L1 preadipocytes were allowed to differentiate into adipocytes in the presence or absence of putrescine. Among the tested polyamine transporters (GPC1, SLC3A2, SLC7A1, SLC7A2, and SLC18B1), both SLC3A2 and SLC7A2 could be involved in the cellular entry of putrescine. As shown in Fig. 6, only the knockdown of SLC3A2 or SLC7A2 was able to significantly decrease the adipogenic effect of putrescine on the expression of adipocyte-specific genes such as PPARγ, FABP4, and adiponectin. However, we could not observed the reduction of the adipogenic effect of putrescine in GPC1-, SLC7A1-, or SLC18B1-knockdown cells, respectively (data not shown). These results indicate that both the polyamine trans-
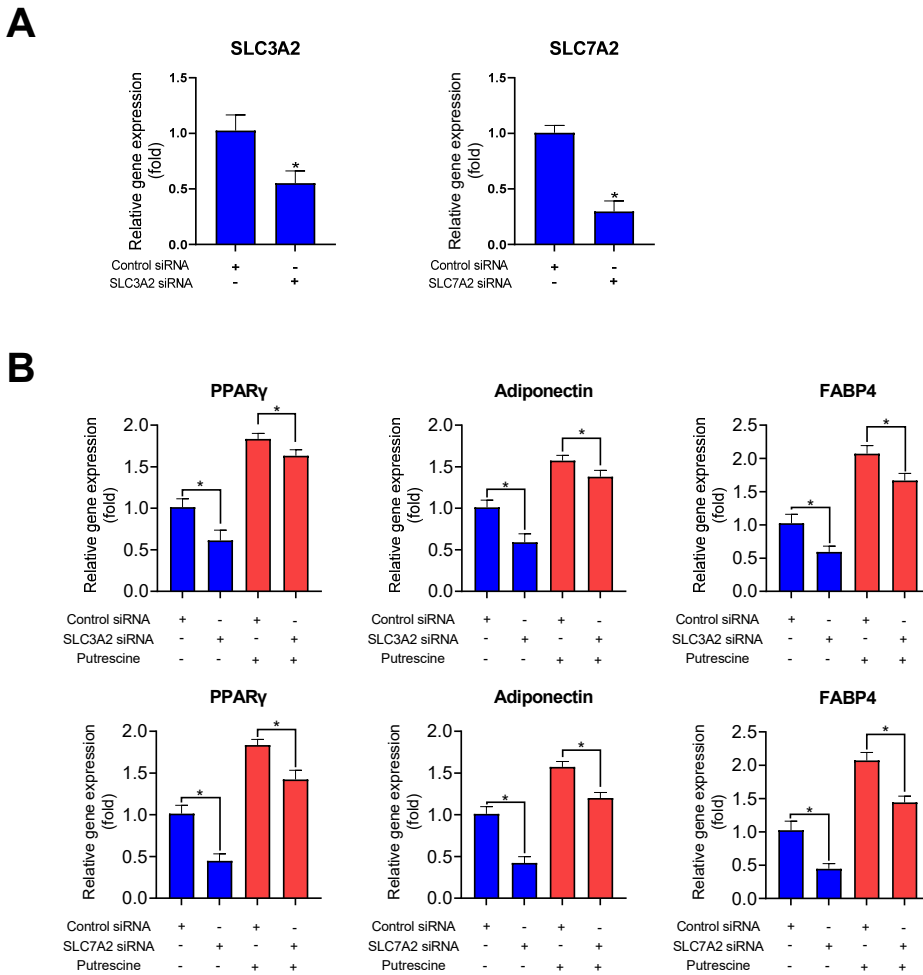
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Fig. 6. The knockdown of polyamine transporters SLC3A2 and SLC7A2 reduces the extent of the putrescine-induced adipogenesis. (A and B) After the transfection of the polyamine transporter siRNA, cells were differentiated into adipocytes in the presence or absence of putrescine, for 2 days. (B) The presence of putrescine (as indicated) and the mRNA expression of adipogenic genes were analyzed by quantitative real-time PCR. All data are presented as the mean ± SEM of two independent experiments (n = 4). *P < 0.05 versus the control group (two-way ANOVA followed by Tukey's post hoc test). PPARγ, peroxisome proliferator-activated receptor gamma; FABP4, fatty acid binding protein 4.

porters SLC3A2 and SLC7A2 facilitate or mediate the entry of exogenous putrescine into the cells, thereby promoting adipocyte differentiation.

Exogenous putrescine stimulates adipocyte differentiation via the modulation of CHOP expression and MCE

In order to investigate the intracellular mechanisms underlying the stimulation of adipocyte differentiation caused by exogenous putrescine, we focused on the effects of putrescine on CHOP expression and MCE. Since it has been reported that the endogenous spermidine synthesis during adipocyte differentiation is required for the downregulation of CHOP in order to allow for the C/EBPβ activation, as well as for activating MCE, we examined the effects of putrescine on CHOP expression and MCE activation. As shown in Fig. 7A, putrescine inhibited the expression of the CHOP mRNA. On the other hand, the mRNA levels of the C/EBPβ, PPARγ, and C/EBPα genes were increased in putrescine-treated cells at the induction period of adipocyte differentiation. In addition, in an attempt to test whether the exogenously-administered putrescine is also able to affect the MCE during adipogenesis, we examined the expression of cell cycle genes and the proliferation rate of 3T3-L1 cells. The mRNA levels of cyclin D1, cyclin E, and cyclin-dependent kinase 2 (CDK2) were found

to be increased by putrescine (Fig. 7B). We also observed that putrescine activates the MCE during adipogenesis, along with an increase of the cell number (Fig. 7C). Taken together, these results suggest that both the downregulation of CHOP and the increment of MCE by exogenous putrescine may contribute to the observed adipocyte differentiation.

DISCUSSION

We have, herein, demonstrated that exogenous putrescine can significantly stimulate adipocyte differentiation by upregulating both adipogenic and lipogenic genes at the early and intermediate stages of differentiation. We have also discovered that the polyamine transporters SLC3A2 and SLC7A2 are involved in the entering of exogenous putrescine into the cells, and that putrescine can subsequently upregulate the expression of the C/EBPβ, PPARγ, and C/EBPα genes in order to promote adipocyte differentiation. Consistent with putrescine, other polyamines such as spermidine and spermine, exerted similar adipogenic effects on the 3T3-L1 adipocyte differentiation (Supplementary Fig. S1).

Polyamines are synthesized within all living cells and can cross the cell membrane (Pegg, 2009). The cellular levels of polyamines are tightly regulated through their import, export,
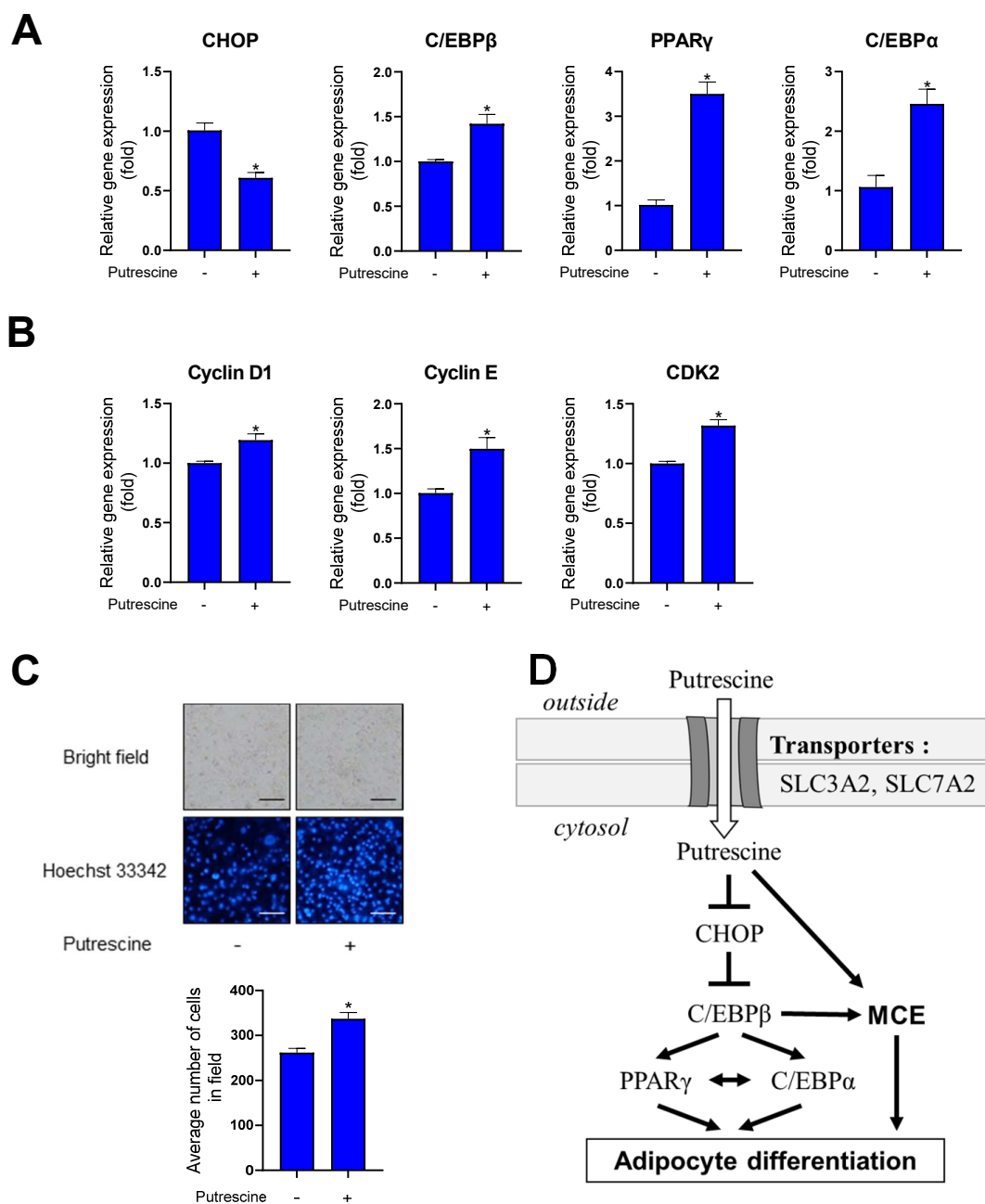
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Fig. 7. Effect of putrescine on CHOP and the cell cycle mRNA expression in early adipogenesis. (A-C) Early adipocytes were proliferated in the presence of 3 mM putrescine. (A) Effect of putrescine on the expression of CHOP-related genes (n = 4). (B) Effect of putrescine on the expression of cell cycle-related genes (n = 4). (C) After staining, cells were counterstained with Hoechst 33342, and were photographed with ImageJ (n = 6). Scale bars = 100 μm. The gene expressions were analyzed by quantitative real-time PCR. All data are presented as the mean ± SEM of two independent experiments (n = 4). *$P < 0.05$ versus the adipocyte group (two-way ANOVA followed by Tukey's post hoc test). (D) Working model for exogenous putrescine-induced adipogenesis. Exogenous putrescine enters the cells through polyamine transporters SLC3A2 and SLC7A2, and subsequently regulates adipocyte differentiation. Once inside the cell, putrescine inhibits CHOP expression in order to allow for the C/EBPβ activation that is required for the expression of the master regulators of adipocyte differentiation (such as PPARγ and C/EBPα); C/EBPβ activation can also promote MCE during adipogenesis. CHOP, C/EBP homologous protein; C/EBPβ, CCAAT/enhancer-binding protein beta; PPARγ, peroxisome proliferator-activated receptor gamma; C/EBPα, CCAAT/enhancer-binding protein alpha; CDK2, cyclin-dependent kinase 2; MCE, mitotic clonal expansion.

synthesis, and catabolism (Zahedi et al., 2022). Polyamine transport and polyamine transporters have been well studied in bacteria, yeast, and *Trypanosoma cruzi* (Abdulhussein and Wallace, 2014; Igarashi and Kashiwagi, 2010; Ramos-Molina et al., 2019). Some transporters such as the SLC family of proteins (SLC3A2, SLC7A1, SLC12A8, SLC22A1, and SLC22A3) as well as ATP13A2 and ATP13A3 have been associated with polyamine transport, but the exact mechanisms underlying the eukaryotic polyamine transport systems are still unclear (Dobrovolskaite et al., 2021; Hamouda et al., 2021; Khan et al., 2021; Madan et al., 2016; Poulin et al., 2012; van Veen et al., 2020). In this study, we have assessed the expression of polyamine transporters GPC1, SLC3A2, SLC7A1, SLC7A2, and SLC18B1 in both preadipocytes and adipocytes. Therefore, we checked whether the stimulating effect of the exogenously-administered putrescine was inhibited by a treatment with the polyamine transporter inhibitor AMXT-1501 during adipocyte differentiation. AMXT-1501 was developed in order to block the polyamine uptake as a competitive inhibitor (Samal et al., 2013). The AMXT-1501 treatment was able to clearly inhibit the exogenous putrescine-induced stimulation of the adipocyte differentiation (Figs. 5B and 5D). Through this, we were able to confirm that the polyamine transporter was involved in the importing of exogenous polyamines. Furthermore, as a result of the undertaken siRNA experiments confirming the involvement of additional polyamine transporters, we were able to confirm the involvement of SLC3A2 or SLC7A2 (Fig. 6).

Both SLC3A2 and SLC7A2 are identified as cationic amino acid transporters. SLC3A2 is a glycosylated heavy chain of cationic amino acid transporters, as a part of a putrescine and acetyl-polyamine efflux system (Pegg, 2009). SLC3A2 with the light chain subunit encoded by SLC7A11 could induce arginine uptake and putrescine efflux (Wang et al., 2022). It was constituted by the cystine/glutamate antiporter system Xc⁻ to mediate extracellular cystine and intracellular glutamate exchange through plasma (Bridges et al., 2012; Lewerenz et al., 2013; Wang et al., 2022). Therefore, the regulation of system Xc⁻mediated cysteine uptake is pivotal in the prevention of ferroptosis, a new form of non-apoptotic cell death (Liu et al., 2021). SLC7A2 is also responsible for the cellular uptake of arginine, lysine, and ornithine. It has been demonstrated that L-arginine uptake through the host cell's SLC7A2 is essential for *Plasmodium* development in the liver (Meireles et al., 2017). Moreover, the previous study reports that loss of SLC7A2 in mice is accompanied by pro-tumorigenic M2 macrophage and exacerbates inflammation-associated colon tumorigenesis (Coburn et al., 2019). Interestingly, the knockdown of SLC3A2 or SLC7A2 itself showed decrease of adipocyte differentiation (Fig. 6B), which could potentially reflect endogenous functions of the transporters. However, further studies are needed.

According to previous studies, eukaryotes use an endosomal system in order to uptake polyamines, in addition to polyamine transporter (Poulin et al., 2012; Roy et al., 2008; Soulet et al., 2004). Therefore, in an attempt to explore whether the endosomal system is involved in polyamine transport, we tried to test the adipogenic effect of exogenous putrescine along with dynasore; an endocytosis inhibitor. Unfortunately,

the adipocyte differentiation was inhibited by dynasore alone (data not shown), so it was not possible to confirm whether the polyamines can be transported into preadipocytes through endocytosis. Further studies are required in order to explore the possibility of an endocytotic uptake of exogenous polyamines, and its molecular mechanism.

C/EBPβ is an early induced transcription factor, and it is essential for adipogenesis. C/EBPβ is required for the execution of the process of MCE as well as for the induction of both PPARγ and C/EBPα. It has been demonstrated that the activation of C/EBPβ is regulated by an heterodimerization with CHOP, and that the dimerization blocks the DNA binding activity, thereby blocking both the MCE and the adipocyte differentiation (Darlington et al., 1998; Soulet et al., 2002; Tanaka et al., 1997; Tang and Lane, 1999). In addition, the overexpression of CHOP-10 prevents adipocyte differentiation (Tanaka et al., 1997). Endogenous polyamine depletion with DFMO has been shown to prevent differentiation and inhibit the second division of the MCE (Brenner et al., 2015). Similarly, we have, herein, shown that the induction of PPARγ and C/EBPα occurs in exogenous putrescine-treated cells, along with an upregulation of the C/EBPβ and a downregulation of the CHOP expression (Fig. 7A). In addition, our results have also revealed that exogenous putrescine can increase the MCE, and can upregulate the expression of genes related to cell cycles (including cyclin D1, cyclin E, and CDK2) (Figs. 7B and 7C). Taken together, these results suggest that the downregulation of CHOP and the promotion of MCE by exogenously-administered putrescine may contribute to adipocyte differentiation (Fig. 7D).

Polyamines are found in the circulation of humans and mice at micromolar concentrations, that are influenced by diet and gut microbiota (Casti et al., 1982; McCubbrey et al., 2022; Ramos-Molina et al., 2019; Soda et al., 2009). Recent studies have suggested that polyamine biosynthesis and transport take place predominantly in the human gut microbiota (Ramos-Molina et al., 2019; Sugiyama et al., 2017). Although this gut microbiota is one of well-known factors involved in the pathogenesis of metabolic disorders such as obesity and type 2 diabetes (Cani et al., 2008; Larsen et al., 2010; Ley et al., 2005; 2006; Pitocco et al., 2020), the involvement of gut microbiota-derived polyamines requires further research. Nevertheless, the dysregulation of the polyamine metabolism has been shown to have an impact on the regulation of the lipid, glucose, and energy homeostasis (Ramos-Molina et al., 2019). Interestingly, altered levels of polyamines have been shown to exist in several tissues of obesity animal models such as adipose tissues, liver, and pancreatic islets (Ramos-Molina et al., 2019). In addition, blood polyamines in obese children have also been found increased (Codoner-Franch et al., 2011; Ramos-Molina et al., 2019). Therefore, we predict that the increased polyamine levels may be implicated in the adipose tissue expandability during obesity. However, further studies are required in order to: (i) test the relevance of the increased polyamine import and accumulation for adipocyte differentiation *in vivo*, (ii) clarify the mechanism(s) of the polyamine entry into the cell, and (iii) understand how exogenous polyamines can be increased in pathophysiological conditions.

AUTHOR CONTRIBUTIONS
J.E. and J.C. performed the experiments. All authors analyzed the data. J.E. and J.B.S. designed the experiments and wrote the article. S.S.S. and J.B.S. performed a critical read of the manuscript. All authors contributed to the article and approved the submitted version.

CONFLICT OF INTEREST
The authors have no potential conflicts of interest to disclose.

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