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Exosomal release through TRPML1-mediated lysosomal exocytosis is required for adipogenesis

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Abstract

The lysosomal Ca^{2+} permeable channel TRPML1 (MCOLN1) plays key roles in lysosomal membrane trafficking, including the fusion of late endosomes to lysosomes and lysosomal exocytosis, both of which are essential for release of exosomes into the extracellular milieu. Multiple lines of evidence indicate that the contents of adipocyte-derived exosomes mediate diverse cellular responses, including adipogenic differentiation. In this study, we aimed to define the potential roles of TRPML1 in lysosomal membrane trafficking during adipogenesis and in exosomal release. In response to adipogenic stimuli, the endogenous TRPML1 expression in OP9 pre-adipocytes was increased in a time-dependent manner, and the acute deletion of TRPML1 reduced lipid synthesis and expression of differentiation-related marker genes. Notably, mature adipocyte-derived exosomes were found to be necessary for adipogenesis and were dependent on TRPML1-mediated lysosomal exocytosis. Taken together, our findings indicate that TRPML1 mediates diverse roles in adipocyte differentiation and exosomal release. Further, we propose that TRPML1 should be considered as a regulator of obesity-related diseases.

Keywords

TRPML1; Adipogenesis; Exosome; Lysosomal exocytosis

1. Introduction

Transient receptor potential mucolipin 1 (TRPML1) is a nonselective Ca^{2+}-permeable cation channel that is mainly expressed in late endosomal/lysosomal membranes, and is involved in the regulation of lysosomal pH and Ca^{2+} mobilization from lysosomes [1]. Loss-of-

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function mutations in TRPML1 causes the lysosomal storage disease, mucolipidosis type IV (MLIV) [2] and cells obtained from MLIV patients show lysosomal enlargement due to the accumulation of undigested materials, including lipids, polysaccharides, and proteins [3], leading to lysosomal dysfunction and cell death. In addition to digestion of cellular materials, lysosomes play crucial roles as a hub for both inward and outward membrane trafficking [4]. Notably, trafficking of lysosomes to and fusion with the plasma membrane, also known as lysosomal exocytosis, mediate various cellular responses, including plasma membrane repair [5], neurite outgrowth [6], and release of lysosomal content [7].

Adipocytes play diverse roles in energy homeostasis by controlling energy consumption [8] and function as a source of proteins and cytokines, which mediate numerous physiological functions [9]. Excessive accumulation of adipocytes, as well as an increase in either their size or number, causes obesity [8], which is a significant risk factor for several diseases, including hypertension, diabetes, and cancer [10]. Therefore, understanding the molecular mechanism underlying adipogenesis is essential for developing new modalities for treating obesity. Accumulating evidence reveal that extrinsic factors such as dexamethasone (DEX) and insulin or increased cAMP are can induce genes regulating adipogenesis *in vitro* [11]. Yet, recent reports demonstrated that, in addition to hormonal cocktails, adipogenesis involves massive secretion of extrinsic factors the form of exosomes [12]. Both pre- and post-adipocytes can secrete exosomes containing microRNAs that regulate communication between cells [13]. Moreover, supplementation of culture media with human adipose tissue extract [14] and miR-450a-5p within rat adipose tissue exosome-like vesicles [13] led to enhancement of adipogenesis and adipogenic differentiation, respectively.

Exosomes originated from multivesicular bodies (MVBs) are packed with microRNAs, mRNAs, and proteins from the parent cells during their formation, and secreted into extracellular milieu by fusion of MVBs with the plasma membrane [15]. Importantly, exosomes play a key role in intercellular communication to modulate numerous crucial pathophysiological processes, including inflammation, tumor invasion, immune responses, and differentiation by transfer of proteins and nucleic acids [16,17]. Recent study showed that down-regulation of NEU1 and accumulation of oversialylated LAMP1 in tumor cells promoted lysosomal exocytosis of hydrolases and exosomes [18]. Therefore, secretion of biomaterials into the extracellular environment by lysosomal exocytosis can be viewed as a mode of intercellular communication in autocrine or paracrine signaling. However, very little is known about the mechanism and physiological roles of intercellular communication by exosomes through lysosomal exocytosis. Considering the endo/lysosomal origin of exosomes and the role of TRPML1 in endo/lysosomal function, in the present study, we examined the role of TRPML1 in exosomal release and adipogenesis.

2. Materials and methods

2.1. Reagents

Minimum essential medium α (MEMα), Opti-MEM alpha, and fetal bovine serum (FBS) were obtained from Gibco (Carlsbad, CA, USA). Total exosome isolation reagent, and lipofectamine RNAiMAX was purchased from Invitrogen (Foster city, CA, USA) and 5-(N,N-Dimethyl) amiloride hydrochloride (DMA), insulin, 3-isobutyl-1-methylxanthine

(IBMX), dexamethasone (DEXA), anti-TRPML1, and oil red O dye were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies against PPARγ was purchased from Santa-Cruz Biotechnology, Inc. (CA, USA). Antibodies against C/EBPα, and β-actin were obtained from Cell Signaling Technology (Beverly, MA, USA). Exosome-depleted FBS and exosome marker antibodies including anti-CD9, anti-CD81, anti-CD63, and anti-Hsp70 were procured from System Biosciences (Palo Alto, CA, USA). Anti-LAMP1 antibody was purchased from Abcam (Cambridge, MA, USA).

2.2. Cell culture and induction of adipocyte differentiation

The bone marrow-derived stromal OP9 cells (ATCC, Manassas, VA, USA) were cultured in MEMα containing 20% FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in 5% CO_2 incubator. To induce differentiation, 1 day post-confluent pre-adipocytes were incubated in differentiation medium containing 10% FBS, 0.5 mM IBMX, 0.25 μM DEXA, 175 nM insulin, 2 mM L-glutamine for 2 days. The medium was then changed to MEMα containing 10% FBS, 2 mM L-glutamine, and 175 nM insulin and the cells were cultured for 3 days.

2.3. Oil red O staining

After the induction of adipocyte differentiation, the cells were washed with cold PBS, fixed at room temperature with 4% formalin for 1 h, and then rinsed with 60% isopropanol. The OP9 cells were stained with oil Red O for 1 h at room temperature and washed 3 times with distilled water. The retained oil Red O dye in the cells was quantified by elution into isopropanol and measured at OD_{520}.

2.4. Quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from the cells using TRIzol (Invitrogen, Foster city, CA, USA) according to the manufacturer's instructions. Briefly, cDNA was synthesized from 1 μg of total RNA using a PrimeScript RT reagent kit (TaKaRa Bio Inc, Kusatsu, Shiga, Japan). Adipocyte differentiation-related gene expression was determined by real-time quantitative PCR using the ABI PRISM 7900 Sequence Detection System and SYBR-Green I (Applied Biosystems, Foster City, CA, USA). The primer sequences for PCR were as follows: PPARγ, 5′-GAAAGACAACGGACAAATCACC-3'; 5′-GGGGGTGATATGTTTGAACTTG-3'; C/EBPα, 5′-TTGTTTGGCTTTATCTCGGC-3'; 5′-CCAAGAAGTCGGTGGACAAG-3'; aP2, 5′-AGCCTTTCTCACCTGGAGA-3'; 5′-TTGTGGCAAAGCCCACTC-3'; TRPML1, 5′-CTCATCACCGGCGCCTAT-3'; 5′-TCACTCTTCTCTGTGCCAGTACCT-3'; C/EBPβ, 5′-ACTACGGTTACGTGAGCCTC-3'; 5′-GGCAGCTGCTTGAACAAGTTC-3'; β-actin, 5′-CCTGTGCTGGTCACCGAGGC-3'; 5′-GACCCCGTCTCTCCGGAGTCCATC-3'. All the results were normalized to the housekeeping gene, β-actin, to control for variation in mRNA concentrations. Relative quantitation was calculated using the comparative $_{\Delta\Delta}$Ct method.

2.5. Western blotting

Each gene expression in protein level was analyzed by western blot following standard procedure. In brief, cells were lysed with RIPA buffer, and samples (10 μg/well) were separated by gel electrophoresis and transferred onto PVDF Membranes (GE Healthcare Life Science, Germany). Following blocking, membranes were, in turn, incubated with primary and HRP-conjugated IgG secondary antibodies. Immunoreactive proteins were detected using an enhanced chemiluminescence (ECL) detection system.

To detect localization of LAMP1 in plasma membrane, plasma membrane was isolated using the Plasma Membrane Protein Extraction kit (Abcam, Cambridge, MA, USA), following the manufacturer's instruction. Briefly, cells were homogenized in homogenization buffer until completely lysed. Homogenates were centrifuged at 700 g for 10 min at 4 °C. Supernatants were transferred to a new tube and centrifuged at 10,000 g for 30 min at 4 °C. The pellets containing total cellular membrane proteins were collected. To purify plasma membrane proteins, pellets were re-suspended in the Upper Phase buffer and added to the Low Phase buffer. Samples were mixed well and incubated on ice for 5 min and centrifuged at 1000 g for 5 min. The upper phase was carefully collected and centrifuged at 10,000 g for 10 min at 4 °C. The plasma membrane pellets were dissolved in 0.5% Triton X-100 in PBS for western blotting. The quantity of LAMP1 in plasma membrane was then measured to quantify translocation of lysosomal protein to the plasma membrane.

2.6. Exosome isolation

Following cell incubation under the designated conditions, culture media was collected and centrifuged at 2000 g for 30 min to remove cells and debris. Supernatants were transferred into new tubes and 0.5 vol of the Total Exosome Isolation reagent (ThermoFisher Scientific, Waltham, MA, USA) were added. Each sample was then incubated overnight at 4 °C, after which the samples were centrifuged at 10,000 g for 60 min at 4 °C. The pellets were suspended in PBS and considered as exosomes. Exosomes were quantified using the BCA protein assay, and 10 μg of exosomes was applied in each experiment.

2.7. Knockdown of TRPML1

The OP9 cells were seeded onto 24-well plate at 70–80% confluency. The next day, cells were transfected with scramble siRNA as a control or recombinant siRNA against TRPML1 (Integrated DNA Technologies, Skokie, IL, USA) using Lipofectamine RNAiMAX, following the manufacturer's protocol. After 72 h incubation, cells were used for the experiments. (siTRPML1: 5′-AGAUUGGCAUUGAGGCAAAGAACCT-3′, 5′-AGGUU CUUUGCCUCAAUGCCAAUCUUC-3′)

2.8. Transmission electron microscopy (TEM)

One drop (10 μL) of sample solution was deposited on a carbon-coated copper grid (Formvar stabilized with 5–10 nm carbon) on 300 mesh (Ted Pella, Inc., Redding, CA, USA) for 50 s and the excess liquid was removed by Whatman qualitative filter paper (Sigma Aldrich, MO, USA). The sample was stained by placing 5 μL of uranyl acetate aqueous solution (0.2 wt%) on the TEM grid. After 50 s, residual staining solution was removed by filter paper and the grid was dried in air for 5 min. The dried specimen was

observed with an energy-filtering transmission electron microscope (LIBRA 120; Carl Zeiss, Oberkochen, Germany) operated at 120 kV and a CCD camera (4000 SP; Gatan, Pleasanton, CA, USA).

2.9. Immunofluorescence microscopy

OP9 cells were seeded onto 35 mm Confocal Dish (SPL Life Science, Gyeonggi-do, Korea) at 80% confluence. Following incubation under designated conditions, cells were fixed with 4% (w/v) paraformaldehyde in PBS for 5 min without membrane-permeabilization step. Cells were then sequentially treated with 10% goat serum, anti-LAMP1 (1:100), and Alexa 568-conjugated secondary antibody (1:500). Fluorescence images were taken by confocal microscopy and analyzed with ImageJ software (NIH, Bethesda, MD, USA; http://imagej.nih.gov/ij/).

2.10. Statistical analysis

Data obtained were analyzed using SPSS version 14.0 (SPSS Inc., Chicago, IL, USA) and are presented as mean ± SEM of the stated number of observations obtained from more than three experiments. Statistical differences were analyzed by one-way ANOVA followed by Tukey's post hoc test. Values of $p < 0.05$ were considered statistically significant (**$p < 0.05$).

3. Results

3.1. Adipogenic stimuli up-regulate endogenous TRPML1 expression in OP9 cells

In this study, we used the bone marrow-derived stromal OP9 cells as a model to study the roles of TRPML1 in adipogenesis. To determine whether TRPML1 is associated with differentiation of OP9 into adipocytes, the cells were induced with a mixture of IBMX, DEXA, and insulin, and adipogenesis was evaluated by examining the time course of expression of adipogenic markers and TRPML1 mRNA and protein levels. The transcript levels, evaluated by qPCR, of adipocyte marker gene PPARγ and of TRPML1 increased gradually from 3 day till 5 day after induction (Fig. 1A). Concomitantly, the protein expression of PPARγ and of TRPML1 also increased during adipogenesis (Fig. 1B). The transcript levels of adipocyte marker gene C/EBPβ significantly increased on 1st and 2nd day of adipogenesis, after which the expression dropped (Fig. 1A). These findings suggest that TRPML1 function, and thus endolysosomal functions, are linked to adipogenesis.

3.2. Acute depletion of TRPML1 impairs differentiation to adipocytes

The expression of TRPML1 was acutely depleted by treating OP9 cells with three concentrations (1, 3 and 10 nM) of TRPML1 siRNAs (siML1). The cells were additionally incubated for 5 days in the presence of adipogenic stimuli. Oil Red O staining indicated that siML1 efficiently reduced the adipogenic stimuli-induced formation of lipid droplets in a dose-dependent manner (Fig. 2A). Next, we determined the effect of TRPML1 knockdown on adipogenic marker gene expression, at both RNA and protein levels. The depletion of TRPML1 resulted in significant reduction of mRNA expression levels of PPARγ, C/EBPα, and aP2 compared with that in scrambled siRNA-treated cells (Fig. 2B). Consistent with these findings, depletion of TRPML1 also reduced the protein expression of PPARγ and

C/EBPα (Fig. 2C). These results indicate that endogenous TRPML1 and its induction in response to adipogenic stimuli plays critical role in adipogenesis *in vitro*.

3.3. TRPML1 is required for exosomal release mediated by lysosomal exocytosis

To determine whether the exosomes isolated from mature adipocytes participate in adipogenic differentiation and the role of TRPML1 in exosomal release, OP9 cells were sequentially induced to differentiate in the presence of adipogenic stimuli for 2 days and then incubated only with insulin for another 2 days. To minimize effects of FBS-derived exosomes, cells were incubated with exosome-depleted FBS-supplemented culture media, and additionally incubated for 2 days. The culture media were then collected, and exosomes were isolated from them. Fig. 3A shows that the isolated adipocyte-derived exosomes (ADEs) present typical morphological features of exosomes. OP9 pre-adipocytes were treated with isolated ADEs in the absence of adipogenic stimuli for 2 days. A significant increase of lipid synthesis was observed in ADE-treated cells compared with cells treated with PBS (Fig. 3B). Further, lipid synthesis and exosomal release was inhibited by treating cells with dimethyl amiloride (DMA), an inhibitor of exosomal release (Fig. 3C). Clear inhibition was observed only with 50 μM DMA, while only a tendency for inhibition was observed with 10–20 μM DMA.

Next, we investigated whether TRPML1-dependent lysosomal exocytosis is pivotal for exosomal release in adipocytes. Fig. 4A depicts the level of exosomal marker proteins, including CD9, CD81, CD63, and Hsp70 in media isolated from the same volume of mock transfected cell and cells transfected with scrambled or TRPML1 siRNA. Notably, depletion of TRPML1 resulted in marked reduction of all four exosomal marker proteins. We then determined whether depletion of TRPML1 attenuated lysosomal trafficking to the plasma membrane. For this, we isolated plasma membrane from mature differentiated adipocytes treated with scrambled or TRPML1 siRNA and observed a close to 50% reduction in surface LAMP1 expression (Fig. 4B). We further confirmed these results by immunostaining differentiated adipogenic cells treated with scrambled or TRPML1 siRNA for LAMP1 to visualize lysosomal LAMP1 at the plasma membrane in unpermeabilized cells. As shown in Fig. 4C, the depletion of TRPML1 in pre-adipocytes resulted in a marked reduction of surface LAMP1. These findings indicate that TRPML1 plays pivotal roles in lysosomal trafficking to the plasma membrane during adipogenesis.

4. Discussion

Intracellular membrane trafficking to lysosomes is indispensable for differentiation of adipocytes, including for the increased autophagic flux to the lysosomes that contributes to cytoplasmic reorganization, degradation of mitochondria by the lysosomes, and formation of lipid droplets [19,20]. Lysosomal membrane trafficking in mature adipocytes participates in acidic lipolysis, in which small lipid droplets are engulfed in autophagosomes and are hydrolyzed in lysosomes upon membrane fusion [21]. These trafficking events involve Ca^{2+}-dependent fusion and fission and are mediated by several membrane proteins, including LAMPs, SNAREs, and ion channels [22-25]. A key protein in membrane fusion and fission is the Ca^{2+} permeable channel TRPML1, which crucially regulates lysosomal pH

and mediates the specific release of Ca^{2+} from lysosomes [1], thus regulating fusion of late endosomes with lysosomes, lysosomal degradation, lysosomal exocytosis, and plasma membrane repair [26-29]. Considering the crucial roles of TRPML1 in lysosomal functions and membrane trafficking, it is surprising that its role in adipogenesis has not been evaluated before.

Our study demonstrated that TRPML1 expression prominently increases in OP9 cells upon adipogenic stimuli. Consistently, depletion of TRPML1 resulted in the reduced differentiation of OP9 cells to adipocytes. Mature adipocytes exhibit unique morphological characteristics, where a single and large lipid droplet occupies most of the cytoplasm. Our findings suggest that pre-adipocytes express TRPML1 in response to adipogenic stimuli, possibly to activate autophagic membrane trafficking and degradation of subcellular organelles, which is required for adipocyte differentiation. Consequentially, deficiency of TRPML1 may result in reduction of differentiated adipocytes and lipid storage.

Exosomes and exosomal miRNAs are key factors regulating cell differentiation [30-33]. miR-450a-5p in exosomes isolated from rat adipose tissue enhances adipogenesis by targeting WISP2, a negative regulator of adipogenesis [13]. Based on these findings, we hypothesized that TRPML1 may modulate exosomal release that is needed for adipogenic differentiation by regulating membrane fusion between MVBs/lysosomes and the plasma membrane. In our study, we found that exosomes act in paracrine and autocrine modes to stimulate adipogenesis. The most noteworthy finding of the present study is that TRPML1 regulated exosomal release by mediating lysosomal exocytosis during adipogenesis. TRPML1, as a Ca^{2+}-permeable channel, controls lysosomal trafficking and fusion oflysosomes with late endosomes, MVBs, autophagosomes, and the plasma membrane [34]. Late endosomes are generally considered as MVBs, and trafficking of late endosomes to the plasma membrane allows the release of exosomes [15]. Although fusion of late endosomes with lysosomes leads to degradation of endosomal content [15], growing evidence in drug-resistant cancer cells indicate that exosomes present in lysosomes are protected from degradation and are released by lysosomal exocytosis, which requires Ca^{2+} release via TRPMLs [35,36].

In summary, the present findings suggest that TRPML1, whose expression is up-regulated by adipogenic stimuli, is crucial for the differentiation of adipocytes by mediating membrane trafficking, exosome formation, and exosomal release. The exosomes derived from lysosomes and released by lysosomal exocytosis have a major role in adipogenesis and lipid metabolism. Furthermore, considering dual roles of lysosomes in lipid metabolism and adipogenesis, our findings suggest that TRPML1 should be considered as a key factor in treating obesity-related diseases.

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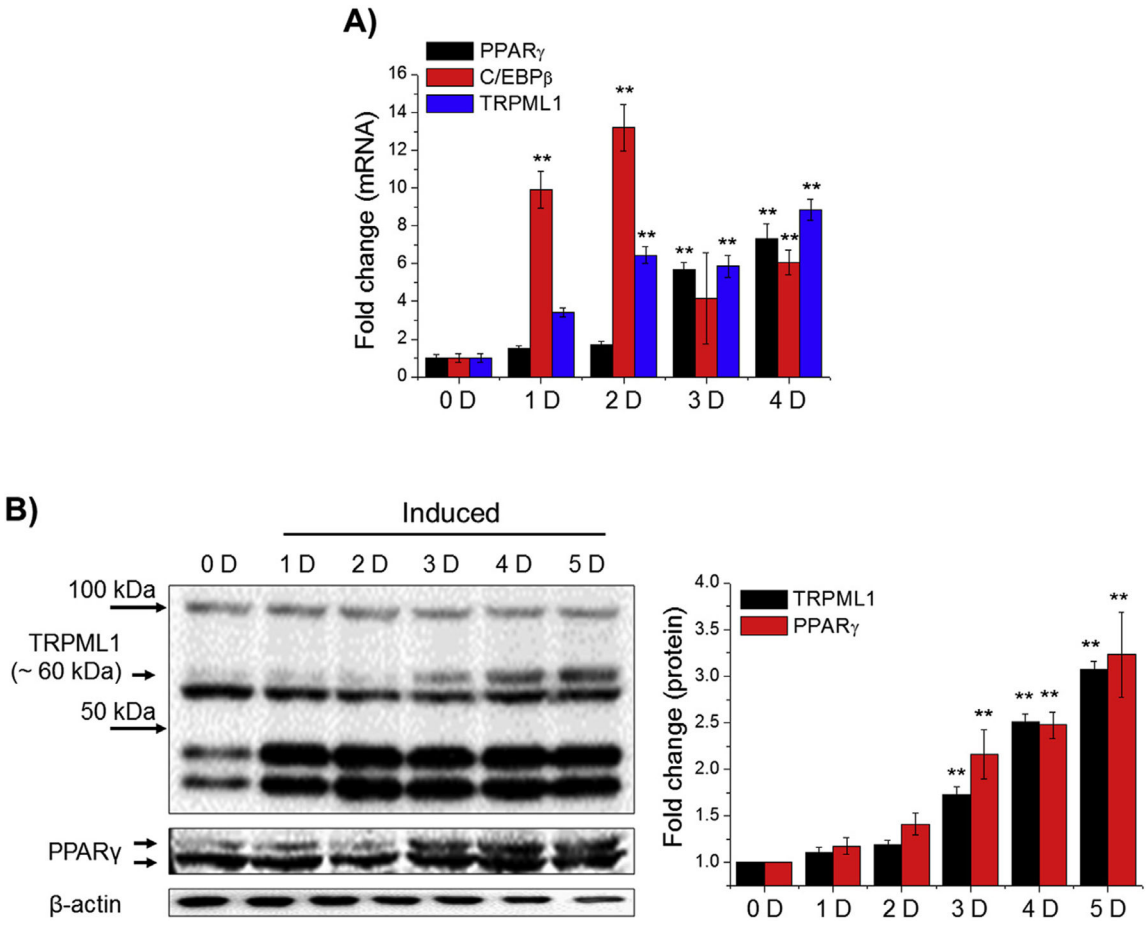

Fig. 1. Expression of endogenous TRPML1 is up-regulated by adipogenic stimuli.

Cells were induced to adipogenic differentiation for indicated days. (A) Quantitative real-time PCR of TRPML1 and adipogenic marker gene expression (C/EBPβ and PPARγ) during adipogenesis. (B) Western blot analysis of TRPML1 and PPARγ expression in response to adipogenic stimuli. Data are expressed as relative fold change of mRNA and protein expression. Graphs represent mean ± SEM from 3 independent experiments. $^{**}P < 0.05$ compared with control group (0 D).

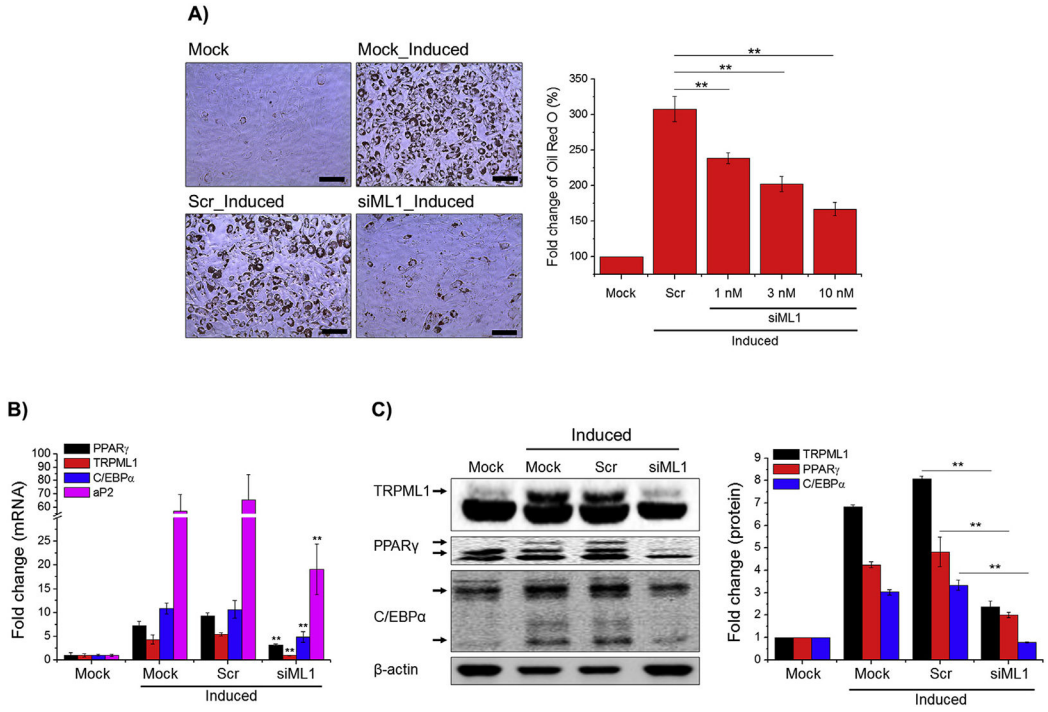

Fig. 2. Effects of TRPML1 depletion on the formation of lipid droplets and adipogenic marker gene expression.

(A) OP9 pre-adipocytes were transfected with mock (transfection reagent only), scrambled siRNA (Scr), and siTRPML1 (siML1) in a dose-dependent manner. Following adipogenic induction, cells were stained with oil red O. (Scale bar, 100 μm). The data in Fig. 2A are expressed as relative fold change of O.D value compared with control group (uninduced mock). (B) Real-time qPCR of TRPML1 and adipogenic marker genes (C/EBPα, aP2 and PPARγ) in each group. (C) Western blot analysis of TRPML1, PPARγ, and C/EBPα expression in each group. Data are expressed as relative fold change of mRNA and protein expression. Graphs represent mean ± SEM from 3 independent experiments. $^{**}P < 0.05$ between the indicated groups.

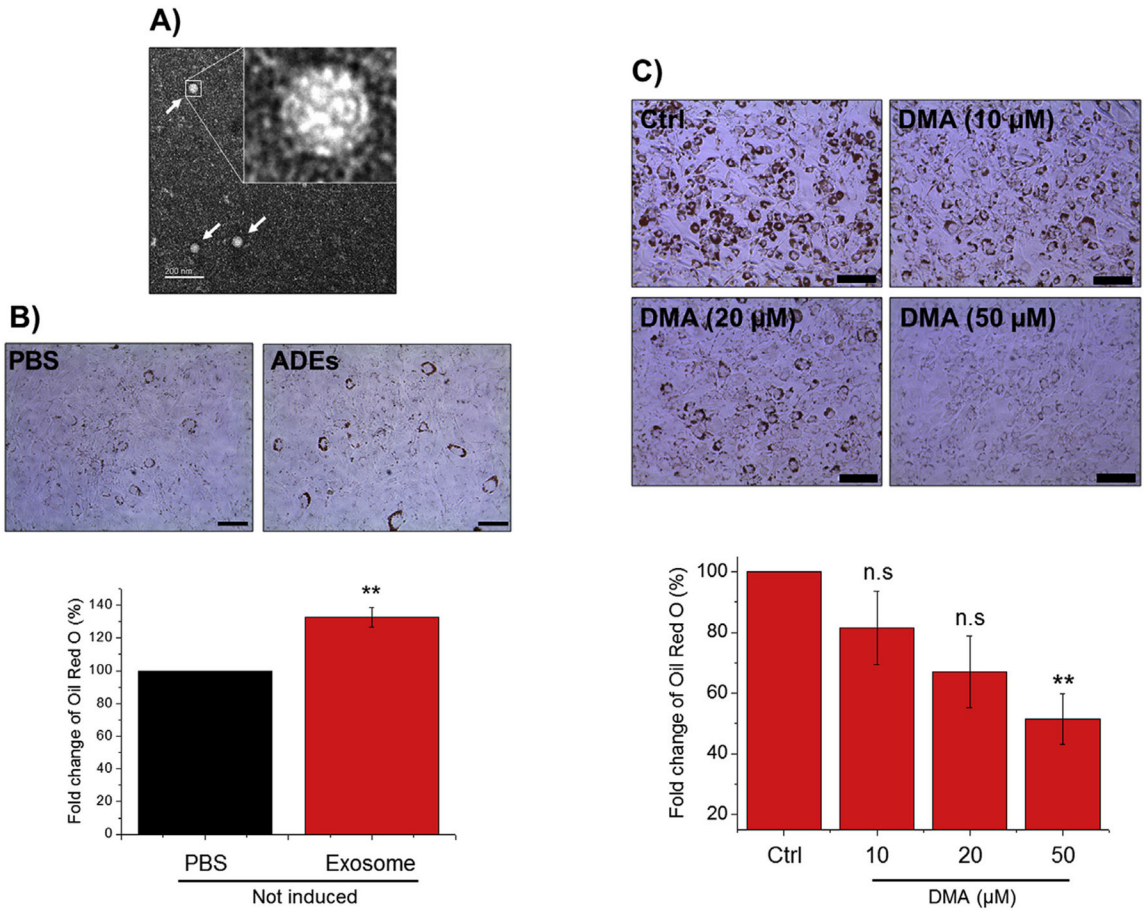

Fig. 3. Regulation of lipid formation by adipocyte-derived exosomes.

(A) Image of exosomes released from mature adipocytes, taken through TEM (Scale bar, 200 nm). Exosomes are marked with white arrows. (B) Adipocyte-derived exosomes (ADEs) suspended in PBS, and 10 μg ADE was incubated with OP9 pre-adipocytes for 2 days. Cells were then stained with oil red O. (Scale bar, 100 μm). (C) OP9 pre-adipocytes were pretreated with DMA in indicated concentrations, and adipogenic differentiation was induced for 4 days, after which cells were stained with oil red O. Data are expressed as fold change of OD value compared with control group (PBS only). $^{**}P < 0.05$ compared with each control group.

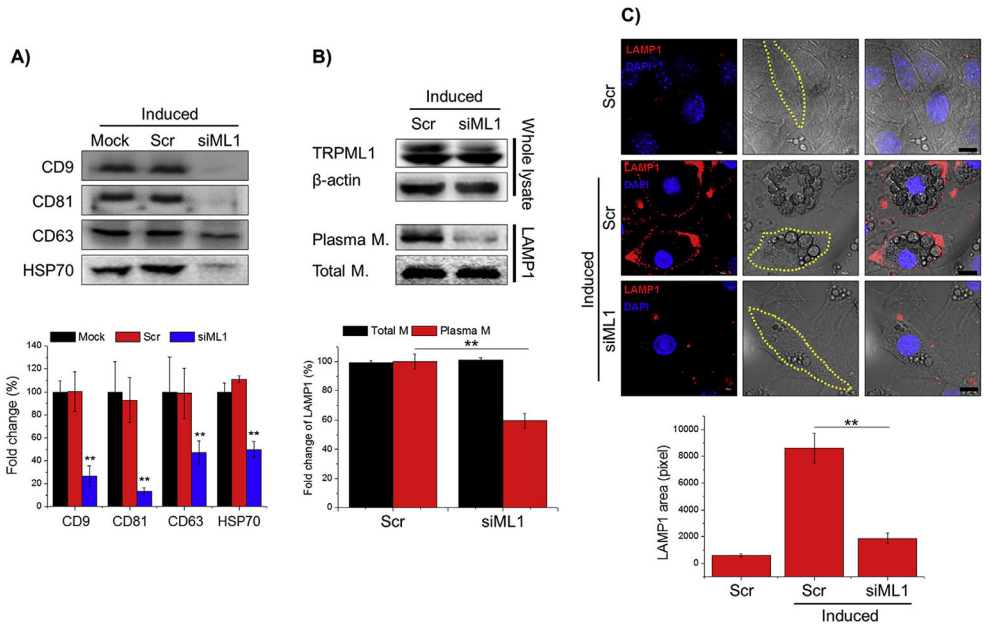

Fig. 4. Exosomal release is regulated by TRPML1-mediated lysosomal exocytosis.

(A) Following induction of adipogenic differentiation, expression of exosomal marker genes (CD9, CD81, CD63, and HSP70) in TRPML1-depleted cells (scramble siRNA; Scr, siTRPML1; siML1). (B) Effects of TRPML1 depletion on localization of LAMP1 in plasma membrane. Plasma membrane (Plasma M) was separated from total membrane (Total M) and used for LAMP1 expression analysis by western blot. (C) Mature adipocytes were immunostained with anti-LAMP1 antibody (Red) and DAPI (Blue), and fluorescence of each image were measured. Data represent mean ± SEM from 3 independent experiments. $^{**}P < 0.05$ compared between indicated groups.