Dexamethasone and rosiglitazone are sufficient and necessary for producing functional adipocytes from mesenchymal stem cells

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Abstract

The final product of adipogenesis is a functional adipocyte. This mature cell acquires the necessary machinery for lipid metabolism, loses its proliferation potential, increases its insulin sensitivity, and secretes adipokines. Multipotent mesechymal stromal cells have been recognized as a source of adipocytes both in *vivo* and *in vitro*. The *in vitro* adipogenic differentiation of human MSC (hMSC) has been induced up to now by using a complex stimulus which includes dexamethasone, 3-isobutyl-1-methylxanthine, indomethacin, and insulin (a classical cocktail) and evaluated according to morphological changes. The present work was aimed at demonstrating that the simultaneous activation of dexamethasone's canonical signaling pathways, through the glucocorticoid receptor and CCAAT-enhancer-binding proteins (*C/EBPs*) and rosiglitazone through peroxisome proliferator-activated receptor gamma (*PPAR-gamma*) is sufficient yet necessary for inducing hMSC adipogenic differentiation. It was also ascertained that hMSC exposed just to dexamethasone and rosiglitazone (D&R) differentiated into cells which accumulated neutral lipid droplets, expressed *C/EBP-alpha, PPAR-gamma, aP2, lipoprotein lipase*, *acyl-CoA synthetase*, *phosphoenolpyruvate carboxykinase*, *adiponectin*, and *leptin* genes but did not proliferate. Glucose uptake was dose dependent on insulin stimulus and high levels of adipokines were secreted (i.e. displaying not only the morphology but also expressing mature adipocytes' specific genes and functional characteristics). This work has demonstrated that (i) the activating *C/EBPs* and *PPAR-gamma* signaling pathways were sufficient to induce adipogenic differentiation from hMSC, (ii) D&R producing functional adipocytes from hMSC, (iii) D&R induce adipogenic differentiation from mammalian MSC (including those which are refractory to classical adipogenic differentiation stimuli). D&R would thus seem to be a useful tool for MSC characterization, studying adipogenesis pathways and producing functional adipocytes.

Keywords: Adipogenesis, adipogenic differentiation, mesechymal stem cell, multipotent stromal cell, functional adipocyte, adipokine

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Introduction

The final product of adipogenesis is a functional adipocyte. This mature cell acquires the necessary machinery for lipid metabolism, loses its proliferation potential, increases its insulin sensitivity, and secretes adipokines.[1–3] Molecular mechanisms behind adipogenic differentiation have been thoroughly studied in immortalized cells already committed to the adipogenic lineage (mouse 3T3-L1 and 3T3F442 cell lines).[4] CCAAT/enhancer binding protein (*C/EBPs*) and peroxisome proliferator activated receptor isoform gamma (*PPAR-gamma*) activation in these preadipocytes triggers the expression of genes related to the mature phenotype[5–8]; exposure to molecules activating *C/EBPs* and *PPAR-gamma* thus results in preadipocyte maturation.[3] To the best of our knowledge, no reports have shown that activating these transcription factors results in primary uncommitted cells' adipogenic differentiation. Multipotent mesechymal stromal cells (also referred to as mesenchymal stem cells—MSC) have been recognized as undifferentiated adipocyte precursors.[9–11] Cells' adipogenic differentiation *in vitro* can be induced by culturing them at confluence and exposing them to a stimuli containing a glucocorticoid (dexamethasone), a phosphodiesterase inhibitor (3-isobutyl-1-methylxanthine—IBMX), a cyclooxygenase inhibitor (indomethacin) and insulin, hereafter referred to as the

classic cocktail (CC).[12] Each CC component activates a different signaling pathway which seems to be relevant for differentiation.[2,13] Due to CC complexity, the molecular events associated with MSC differentiation into the adipogenic lineage have been difficult to elucidate.[14–19] The adipocyte-like cells produced from CC-exposed MSC have been mainly characterized according to their morphology (the presence of oil droplets and fusiform transition to spherical form) and gene and protein expression of adipogenesis terminal phase markers.[9,12,14,16,20,21] However, it is unknown whether adipocytes differentiate *in vitro* into functional cells.

Our work was aimed at developing a simple differentiation stimulus which would allow functional adipocytes to be produced from human MSC (hMSC). This involved evaluating the dependence of hMSC adipogenic differentiation on the activation of dexamethasone's canonical signaling pathways through the glucocorticoid receptor (GR) and the rosiglitazone pathway through *PPAR-gamma*, assessing the presence and abundance of Oil Red O and Nile Red-stained cells, the subcellular localization of *C/EBP-alpha* and *PPAR-gamma*, and their mRNA levels in dexamethasone and rosiglitazone (D&R)-exposed cells in the absence or presence of inhibitors for both signaling pathways. The degree of functionality of adipocytes produced from D&R-exposed hMSC was evaluated by assessing their gene profile, proliferation potential, insulin sensitivity, and adipokine secretion. It was also ascertained whether mouse, rat, hamster, rabbit, dog, and/or human MSC exposed to D&R led to adipocyte-like cell production by assessing the presence and abundance of Oil Red O- and Nile Red-stained cells.

Materials and methods

MSC isolation, expansion, and characterization

hMSCs were isolated from remaining material after heparinized aspirates had been obtained from normal donors undergoing bone marrow harvest for allogeneic transplant. MSCs from mice (C57BL/6 strain), rats (Wistar), hamsters (Syrian Golden Hamster), and rabbits (New Zealand) were isolated from their femurs and tibias. MSCs from dogs (German shepherd breed) were isolated from heparinized aspirates taken from harvested bone marrow. The Clínica Alemana's Ethics Committee (Santiago, Chile) approved using human leftover material for research. The Medicine Faculty's Ethics Committee at the Clínica Alemana, Universidad del Desarrollo, approved the animal protocols. Bone marrow samples were diluted with phosphate-buffered saline (PBS, Gibco, Grand Island, NY) and spun at $400 \times g$ for 7 min. Cells were seeded at 1×10^6 nucleated cells/cm^2 density in alpha-minimal essential medium (alpha-MEM, Gibco, Grand Island, NY) supplemented with 10% (v/v) selected fetal bovine serum (HyClone, Logan, UT) and 80 mg/mL gentamicin (Laboratorio Biosano, Chile), hereafter referred to as alpha-10 medium. Non-adherent cells were removed one day later by replacing the culture medium. When fibroblastic-like cells foci were confluent, cells were detached with 0.25% (w/v) trypsin, 2.65 mM EDTA (Gibco, Grand Island, NY), and subcultured at 7×10^3 cells/cm^2 density. Cultures were kept in a humidified atmosphere containing 5% (v/v) CO_2 at 37°C. Adherent cells were immunophenotyped after two passages and characterized according to their adipogenic, chondrogenic, and osteogenic differentiation potential.[22,23]

MSC exposure to differentiation stimuli

MSCs were seeded at 2.5×10^4/cm^2 density in alpha-10 medium. The medium was replaced one day later with alpha-10 supplemented with CC (1 μM dexamethasone [Sigma-Aldrich, Germany], 100 μg/mL IBMX (Calbiochem, La Jolla, CA), 100 μM indomethacin [Sigma-Aldrich, St. Louis, MO], and 0.2 U/mL insulin [Sanofi-Aventis, Frankfurt, Germany]), separate CC components, or 1 μM dexamethasone and 10 μM rosiglitazone (D&R, patent pending—PCT/CL2012/000076). Differentiation stimuli were replaced every two days. Cultures were maintained in a humidified atmosphere containing 5% (v/v) CO_2 at 37°C.

Assessing adipogenic differentiation

Cells were stained with 60% (w/v) Oil Red O (Sigma-Aldrich, Germany) in isopropanol for 1 h at room temperature (RT). Once washed, cells were observed and photographed by light microscope (ECLIPSE TS100. Nikon, Japan); alternatively, cells were trypsinized, stained with 1 mg/mL Nile Red (Sigma-Aldrich, St. Louis, MO) for 30 min at RT and analyzed by flow cytometry (CYAN ADP, Dako Cytomation, Carpinteria, CA). Summit V 4.3 software was used for data acquisition and processing.

Cell viability

Cell viability was determined based on trypan blue dye exclusion criteria. Cells were trypsinized and a representative aliquot was diluted two times with 0.4% trypan blue (w/v). The number of total cells and dead cells was determined in a Neubauer chamber. CV (%) = (total cells–dead cell/total cells) × 100.

C/EBP-alpha and PPAR-gamma subcellular localization

Cells were fixed with 4% (v/v) paraformaldehyde (Merck, Germany) for 10 min at 4°C, washed, and permeabilized with 0.2% (v/v) Triton X-100 in PBS for 15 min at RT. Once blocked with PBS containing 5% (w/v) BSA and 0.1% (v/v) Triton X-100 for 1 h at RT, cells were washed and incubated with 1/50 rabbit polyclonal IgG anti-CEBP-alpha (Santa Cruz Biotechnology Inc, Santa Cruz, CA) or 1/50 chicken polyclonal IgY anti-PPAR-gamma (Gen Way Biotech Inc, San Diego, CA) overnight at 4°C. After three washes, cells were incubated with 1/100 goat anti-rabbit IgG serum conjugated with Alexa-fluor488 (Molecular Probes, Eugene, OR) or 1/100 donkey anti-chicken IgY serum conjugated with Cy2 (Jackson Immune Research, Baltimore, PA) for 1 h at RT. Cells were washed again, counterstained with Topro-3 (Invitrogen, Carlsbad, CA), mounted with mounting medium (DAKO, Carpinteria,

CA), observed and photographed by LSM 510 confocal microscope (Zeiss, GmbH, Germany).

GR and PPAR-gamma inhibition

hMSCs were seeded at $2.5 \times 10^4/cm^2$ density in the presence of D&R, with or without 10 μM RU486 (Sigma-Aldrich, St. Louis, MO), a GR antagonist,[24] and/or 10 μM GW9662 (Sigma-Aldrich, St. Louis, MO), an inhibitor of PPAR-gamma[25] D&R + inhibitors (D&R + I); the medium (D&R + I) was replaced every two days. RU486 and GW9662 were added separately or together at the indicated concentrations each 24 h. Cultures were maintained in a humidified atmosphere containing 5% (v/v) CO_2 at 37°C.

Adipocyte-specific gene expression

Total RNA was isolated from cells using Trizol reagent (Invitrogen, Carlsbad, CA) and quantified by absorbance at 260 nm. One microgram total RNA was reverse transcribed with 200 U M-MLV reverse transcriptase (Promega, Madison, WI) and 300 pmol oligo-dT. Real-time PCR was performed in 20 μL final volume containing 50 ng cDNA, PCR LightCycler-DNA Master SYBRGreen reaction mix (Roche, Indianapolis, IN), 4 mM $MgCl_2$, and 0.5 μM each specific primer (Supplementary Table S1) using a LightCycler thermocycler (Roche, Indianapolis, IN). Controls without reverse transcription were included to ensure that amplification was from mRNA and not from genomic DNA. Amplicons were characterized according to their melting temperature, determined by LightCycler thermocycler, and after their size had been evaluated after being electrophoresed on agarose gel (Supplementary Table S1). Each target gene's mRNA level was standardized against the *GAPDH* mRNA level for each sample. The ΔCt method was used for mRNA quantification, expressed as arbitrary units (a.u.).[26]

Assessing proliferation potential

Cells were trypsinized and seeded at $1 \times 10^4/cm^2$ density in alpha-10 medium. One day later the medium was supplemented with 25 μM BrdUrd (or not); two days later the cells were trypsinized, fixed, permeabilized, and stained with anti-BrdUrd-APC antibody, following the manufacturer's instructions (BrdU Flow kit, BD Pharmingen, San Diego, CA). The cells were then stained with Nile Red and analyzed by flow cytometry (CYAN ADP, Dako Cytomation, Carpinteria, CA). Summit V 4.3 software was used for data acquisition and processing.

Determining insulin sensitivity

Culture media were replaced by alpha-MEM. The cells were washed with PBS 4 h later and incubated without or with 0.1, 1, or 10 nM insulin for 30 min at 37°C. Culture media were replaced by PBS containing 4 mM 2-deoxyglucose (2-DG) and 2 μCi/mL [H^3]dT 2-DG and cells were incubated for 1 min at 37°C. Glucose transport was stopped by washing with cold PBS and freezing samples at −20°C. The cells were then lysed with formic acid (0.5 N, for 1 h at RT and for 30 min at RT). Lysates were diluted in 2 mL biodegradable scintillation solution. Radioactivity was measured on a LKB Rackbeta 1217 scintillation counter (LKB Instruments, INC, Rockville, MD) and standardized against protein concentration.[27]

Quantifying adipokine secretion

Culture media were replaced by alpha-MEM; the conditioned media were collected two days later. The level of adiponectin secreted by cells was determined by Quantikine human adiponectin/Acrp30 immunoassay and leptin level by Quantikine human leptin immunoassay (R&D System Inc, Minneapolis, MN).

Statistical analysis

The data have been presented as mean ± SEM. Analysis of variance (ANOVA) was used for multiple group comparisons, followed by Tukey's *post hoc* test. $p < 0.05$ was considered to be statistically significant.

Results

D&R-induced adipogenic differentiation of hMSC

While D&R-exposed hMSC differentiated into adipocytes, cells exposed to alpha-10 medium supplemented with the vehicle (DMSO), dexamethasone, rosiglitazone, IBMX + rosiglitazone, or indomethacine + rosiglitazone remained undifferentiated (Figure 1(a)). Regarding adipogenic differentiation efficiency, a significant difference between CC and D&R (45 ± 5% *cf* 25 ± 3%, respectively) was observed regarding the production of cells accumulating neutral lipid droplets in their cytoplasm (Figure 1(b)). However, D&R triggered the adipogenesis of all hMSC samples tested (7/7).

D&R-induced adipogenic differentiation of other mammalian MSC

MSC from mice, rats, hamsters, rabbits, and dogs D&R exposed produced adipocytes whereas CC scarcely promoted mouse and dog MSC differentiation in the same conditions (Figure 2(a) and (b)).

The simultaneous activation of dexamethasone canonical signaling pathways through GR and C/EBPs and rosiglitazone through PPAR-gamma was necessary for inducing hMSC adipogenic differentiation

C/EBP-alpha and *PPAR-gamma* were found in the nucleus of differentiated cells when D&R-exposed (Figure 3(a)). Both transcription factors remained in hMSC cytoplasm in the presence of the GR inhibitor, regardless of whether they were D&R exposed. GW9662 (used at concentrations selectively inhibiting *PPAR-gamma*) reduced the number of cells in which *C/EBP-alpha* and *PPAR-gamma* became located in the nucleus (Figure 3(a)). Regarding *C/EBP-alpha* and *PPAR-gamma* gene expression, it was observed that exposure to RU486 and/or GW9662 resulted in significant inhibition of *C/EBP-alpha* by day 7 following treatment, while no significant changes were observed regarding *PPAR-gamma* expression levels at any of the times studied here

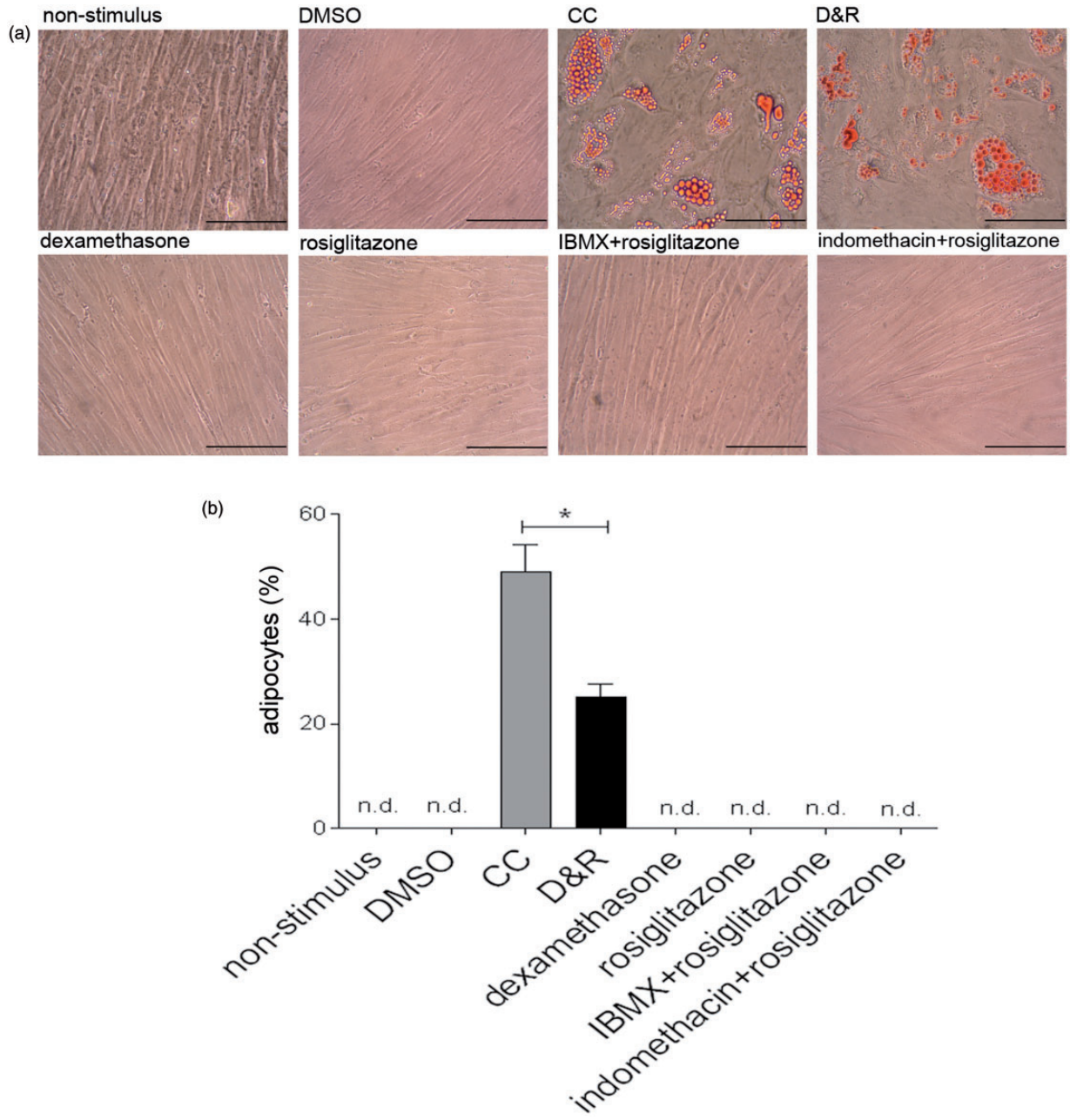

Figure 1 D&R-induced hMSC adipogenic differentiation. hMSCs were exposed to non-stimulus, DMSO, CC, D&R, dexamethasone, rosiglitazone, IBMX + rosiglitazone, or indomethacin + rosiglitazone. Adipocyte presence and abundance were assessed on day 14 by Oil Red O (a) and Nile Red (b) staining, respectively. Representative data regarding seven different hMSC donors. Cell viability >95%. Scale bar: 100 μm. n.d. below the assay detection limit.*: $p < 0.05$ (ANOVA, Tukey). (A color version of this figure is available in the online journal.)

(Figure 3(b)). GR inhibition resulted in the complete abolishment of hMSC adipogenic differentiation (Figure 4(a) and (b)). Partial inhibition caused by D&R-triggered differentiation was observed when *PPAR-gamma* was blocked by GW9662 (Figure 4(a) and (b)); similarly, the genes associated with the adipocyte mature phenotype were not expressed or were so at very low levels when GR or *PPAR-gamma* was inhibited, respectively (Supplementary Figures 1 and 2). The simultaneous activation of GR and *C/EBPs* and *PPAR-gamma* was thus necessary for inducing D&R-triggered hMSC adipogenic differentiation.

Adipocytes produced from D&R-exposed hMSC were functional

As seen in Figure 5, hMSC exposed to CC or D&R gradually expressed genes related to adipogenic commitment (*C/EBP-alpha* and *PPAR-gamma*), lipid storage (*adipocyte protein2* [*aP2*] and *lipoprotein lipase* [*LPL*]), triacylglycerol metabolism (*acyl-CoA synthase* and *phosphoenolpyruvate carboxykinase (PEPKC)*), and adipokines (*adiponectin* and *leptin*), reaching maximum expression on day 14. Most mRNAs assessed were more abundant in hMSC exposed to CC than to D&R; such difference correlated with the higher percentage of adipocytes found in the former

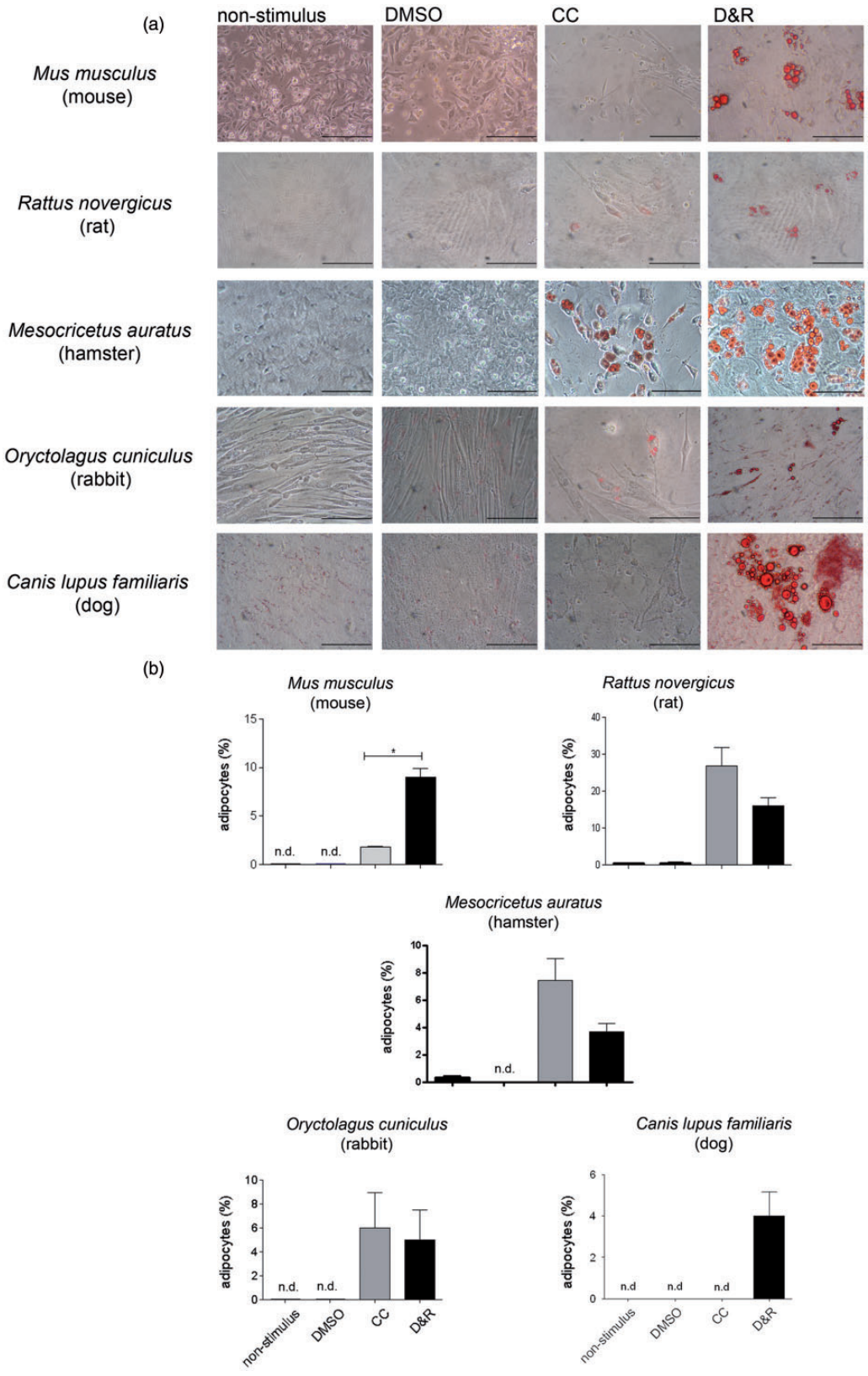

Figure 2 D&R induced the adipogenic differentiation of MSC from different mammalian species. MSCs isolated from mice, rats, hamsters, rabbits, and dogs were exposed to non-stimulus, DMSO, CC, or D&R. Adipocyte presence and abundance were assessed on day 14 by Oil Red O (a) and Nile Red (b) staining, respectively. Representative data regarding seven different hMSC donors. Cell viability >95%. Scale bar: 100 μm. n.d. below the assay detection limit.*: $p < 0.05$ (ANOVA, Tukey). (A color version of this figure is available in the online journal.)

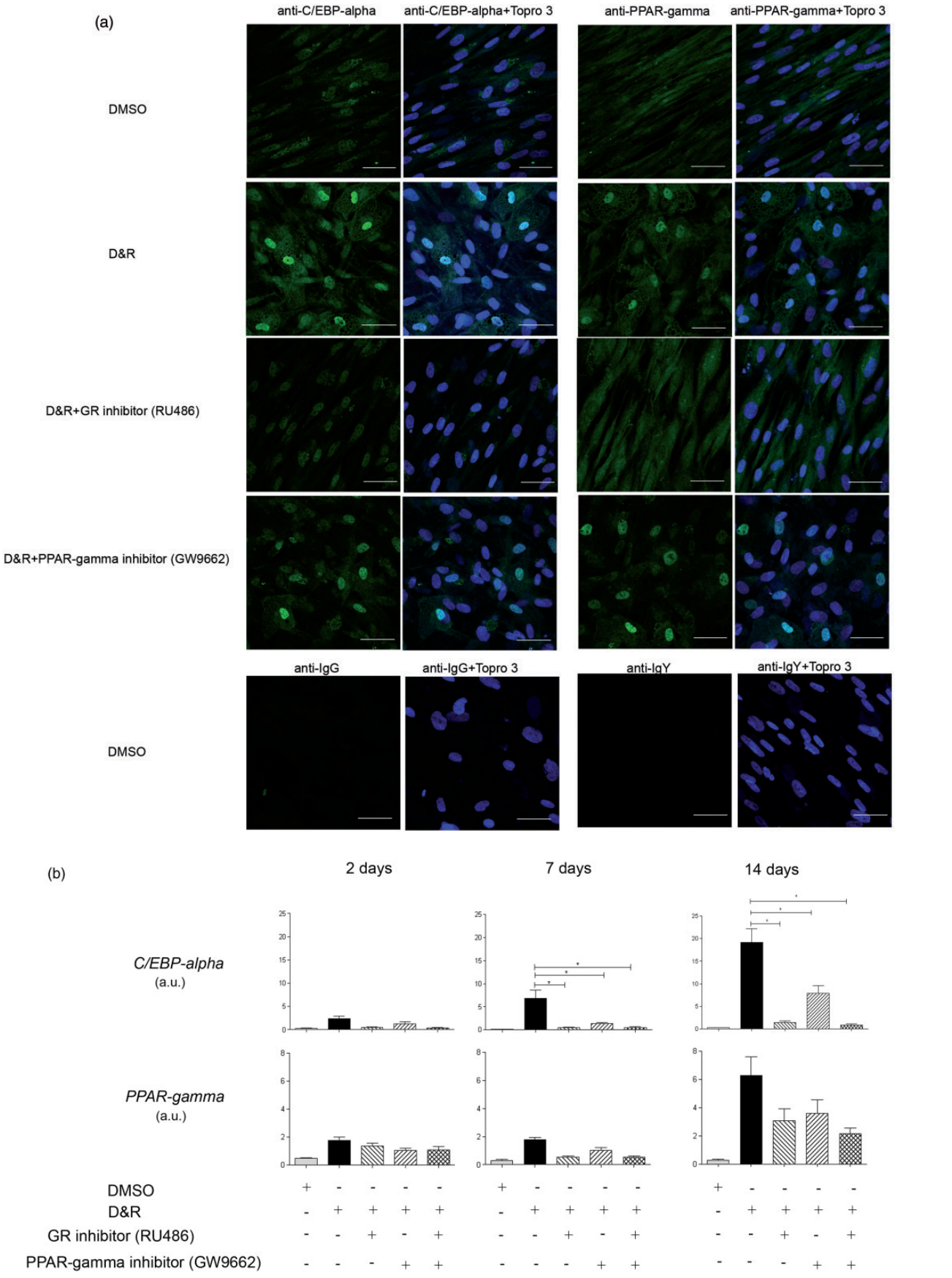

Figure 3 Dexamethasone canonical signaling pathway through GR and rosiglitazone signaling pathway through *PPAR-gamma* became activated when hMSCs were D&R exposed. hMSCs were D&R exposed in the absence or presence of a GR inhibitor (RU486) and/or a *PPAR-gamma* inhibitor (GW9662). *C/EBP-alpha* and *PPAR-gamma* subcellular localization was assessed by immunocytofluorescence on day 14 (a). Representative data regarding seven different hMSC donors. *C/EBP-alpha* and *PPAR-gamma* mRNA levels were assessed on days 2, 7, and 14 by RT-PCR. (b) Scale bar: 50 μm.*: $p < 0.05$ (ANOVA, Tukey). (A color version of this figure is available in the online journal.)

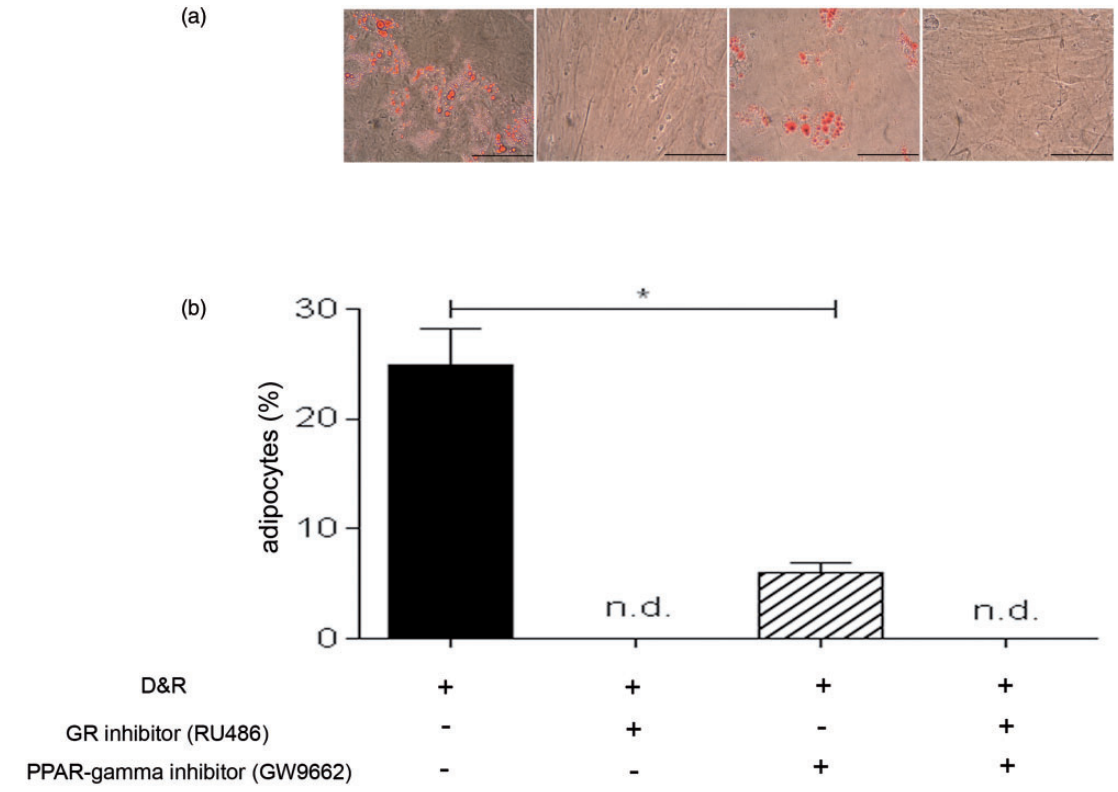

Figure 4 Activating dexamethasone canonical signaling pathways through GR and rosiglitazone through *PPAR-gamma* was necessary to induce hMSC adipogenic differentiation. hMSCs were D&R exposed in the absence or presence of a GR inhibitor (RU486) and/or a *PPAR-gamma* inhibitor (GW9662). The presence and abundance of adipocytes were assessed on day 14 by Oil Red O (a) and Nile Red (b) staining, respectively. Representative data regarding seven different hMSC donors. Cell viability >95%. Scale bar: 100 μm. n.d. below the assay detection limit.*: $p < 0.05$ (ANOVA, Tukey). (A color version of this figure is available in the online journal.)

(Figure 1). Nonetheless, *adiponectin* levels were similar, and those of *leptin* were significantly higher in D&R-exposed hMSC compared to cells exposed to CC. When the data were standardized against adipocyte percentage, the difference was two and 250 times higher for *adiponectin* and *leptin*, respectively. When proliferation potential was assessed, it was observed that regardless of the stimulus used, adipocytes produced from hMSC (Nile Red positive cells) were unable to proliferate (BrdUrd negative) (Figure 6); however, undifferentiated hMSC proliferated in the same culture conditions (Nile Red negative, BrdUrd positive). Regarding insulin sensitivity, our results showed that undifferentiated hMSC did not incorporate a glucose response to insulin (Figure 7). The same was observed for adipocytes produced with CC. Furthermore, glucose uptake did not significantly vary at different insulin concentrations in these cells. Interestingly, D&R-exposed hMSC incorporated glucose in response to insulin in a dose-dependent way. It was also observed that undifferentiated cells secreted neither adiponectin nor leptin and hMSC exposed to CC secreted adiponectin and marginally, leptin (Figure 8). Adipocytes produced from D&R-exposed hMSC secreted both adipokines. Data standardized against adipocyte percentage revealed that D&R resulted in adiponectin- and leptin-secretion levels four and 800 times higher than CC, respectively.

Discussion

Adipogenesis involves two steps: the commitment of an undifferentiated stem cell to the adipogenic lineage followed by the maturation of a preadipocyte into a functional adipocyte.[1,3,28] While MSCs are recognized as a source of adipocytes *in vivo* and *in vitro*, the characterization of the cellular and molecular events leading to their adipogenic differentiation has been poorly studied as have the functional stages (gene expression profile, proliferation potential, insulin sensitivity, and adipokine secretion) of the cells so produced. This paper has shown that exposure to D&R, but not to each of its components separately, induced hMSC adipogenic differentiation (Figure 1(a) and (b)). These results were consistent with Ninomiya's work which showed that rosiglitazone treatment alone was not sufficient for inducing functional adipocytes from hMSC.[20] Furthermore, an attempt was made to ascertain whether D&R was sufficient to induce adipogenic differentiation in MSC obtained from other mammals commonly used as research models. The results showed that D&R induced adipogenic differentiation in all the species tested here, even in those where CC appeared to be refractory (Figure 2(a) and (b)).

D&R-induced adipogenesis in hMSC depended on *C/EBP-alpha* and *PPAR-gamma* activation (Figures 3 and 4). This was consistent with previous report which

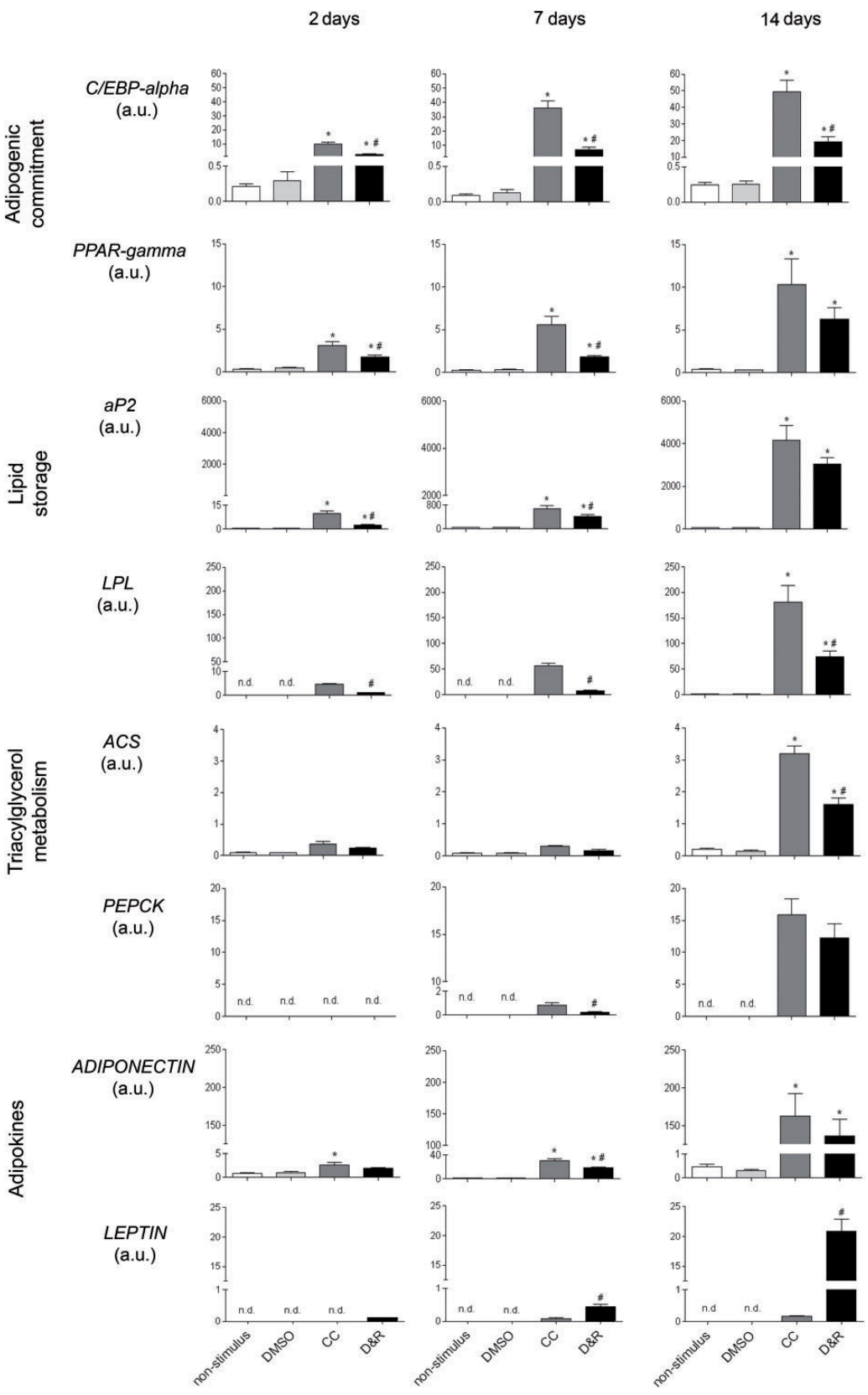

Figure 5 Adipocytes produced from D&R-exposed hMSC expressed functional adipocyte-specific genes. hMSCs were exposed to non-stimulus, DMSO, CC, or D&R. Adipocyte marker gene mRNA levels were assessed by RT-PCR on days 2, 7, and 14. Representative data regarding seven different hMSC donors. n.d. below the assay detection limit.*: $p < 0.05$, versus DMSO. #: $p < 0.05$, versus CC (ANOVA, Tukey)

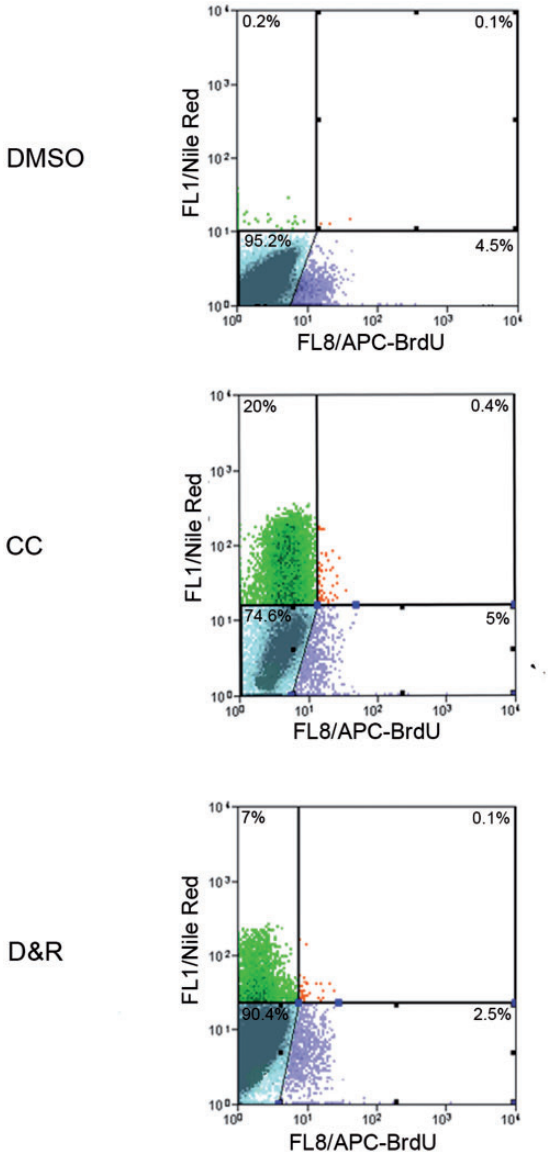

Figure 6 Adipocytes produced from D&R-exposed hMSC had no proliferation potential. hMSCs were exposed to DMSO, CC, or D&R. Cells were subcultured on day 14 in conditions which allowed proliferation; cells were incubated with BrdUrd one day later, and immunocytofluorescence was performed two days later. Representative data regarding seven different hMSC donors.*: $p < 0.05$ (ANOVA, Tukey). (A color version of this figure is available in the online journal.)

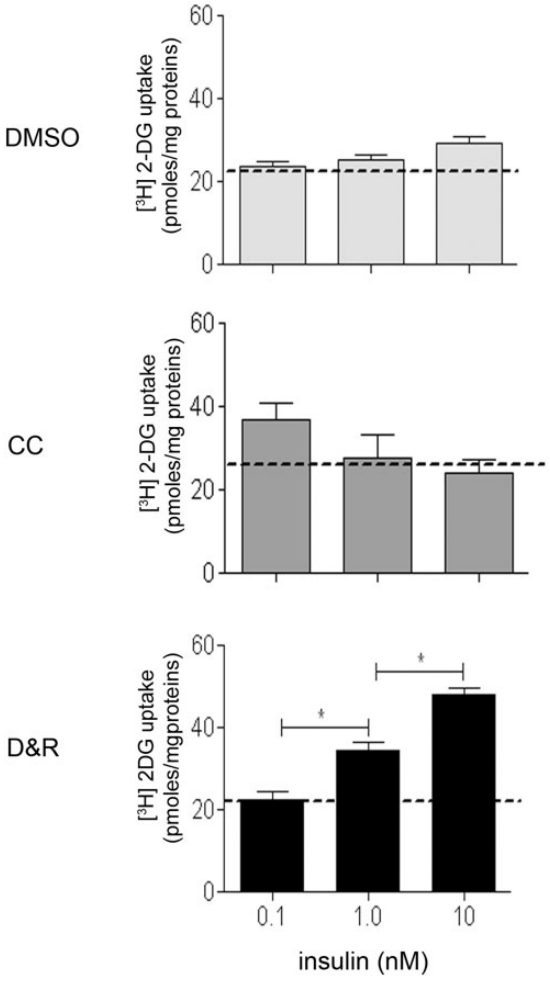

Figure 7 Adipocytes produced from D&R-exposed hMSC were sensitive to insulin. hMSCs were exposed to DMSO, CC, or D&R. Cells were treated with increasing concentrations of insulin on day 14 and [H^3]dT 2-DG uptake was assessed. Representative data regarding three different hMSC donors. Dotted lines indicate base uptake for each condition.*: $p < 0.05$ (ANOVA, Tukey)

have shown *in vivo* and *in vitro* that *C/EBPs* and *PPAR-gamma* are crucial in inducing and maintaining the adipose phenotype and also with the fact that these transcription factors are activated in MSC induced to the adipogenic lineage in the presence of CC.[9,13,18,19,29] Since hMSC exposed to dexamethasone did not differentiate into adipocytes (Figure 1(a) and (b)), *C/EBP's* activation was not sufficient to trigger adipogenesis in uncommitted cells and required the simultaneous activation of *PPAR-gamma*. Further support for the fact that D&R activates dexamethasone and rosiglitazone's canonical signal pathways was provided by the increased expression of both transcription factors in the nucleus and hMSC became differentiated into adipogenic lineage (Figure 3(a)) accompanied by a significant increase in their gene expression (Figure 3(b)), whereas *C/EBP-alpha* and *PPAR-gamma* expression decreased in the presence of the GR inhibitor (RU486) and was preferably located in the cytoplasm of undifferentiated hMSC. This led to hypothesizing that *C/EBP-alpha* and *PPAR-gamma* quantification in the nuclear fraction of D&R-exposed hMSC could complement the results observed in immunocytofluorescence experiments.

Furthermore, interdependency between these two signal pathways was revealed since *PPAR-gamma* mRNA levels did not increase in the presence of the GR inhibitor (Figure 3(b)). Interestingly, while GR inhibition led to a complete blockade of adipogenic differentiation from hMSC (Figure 4(a) and (b)), *PPAR-gamma* inhibition led to a partial reduction of such differentiation (Figure 4(a) and (b)). It has been reported that dexamethasone promotes dissociation and degradation of the mSin3A/HDAC1 complex (repressing *C/EBP-beta*) in 3T3-L1 cells.[30,31] Activated *C/EBP-beta* triggered *C/EBP-alpha* and *PPAR-gamma* expression, inducing and maintaining the adipose phenotype.[32,33]

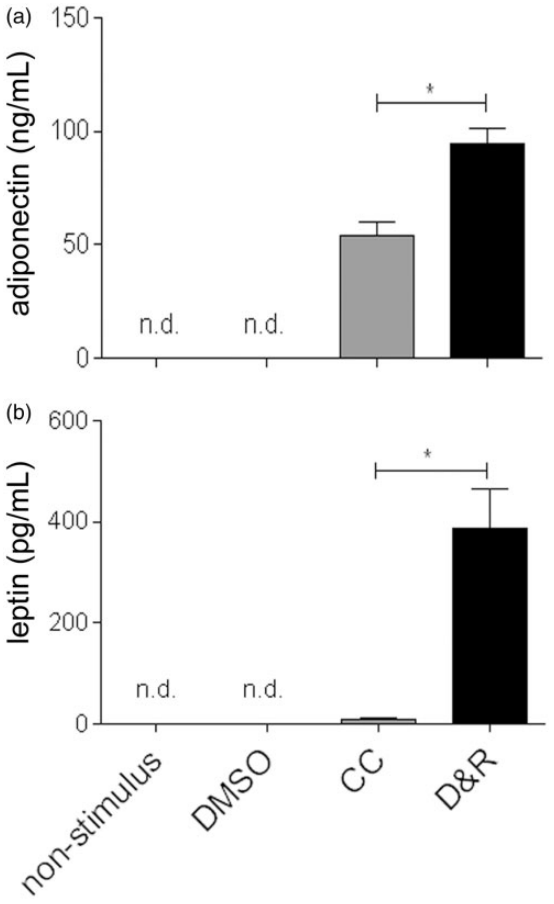

Figure 8 Adipocytes produced from D&R-exposed hMSC secreted adiponectin and leptin. hMSCs were exposed to non-stimulus, DMSO, CC, or D&R. Adiponectin (a) and leptin (b) levels in conditioned media were assessed by ELISA on day 14. Representative data regarding seven different hMSC donors.*: $p < 0.05$ (ANOVA, Tukey)

The above and the results obtained here led to suggesting that hMSC adipogenic differentiation could involve a mechanism similar to that described in 3T3-L1 cells. Consequently, *C/EBP-alpha* inhibition triggered by RU486 led to an early blocking of the *C/EBP-beta* and *C/EBP-delta*-mediated signaling pathway cascade, *C/EBP-delta* having been described as a complementary transcription factor regarding *C/EBP-beta* function, thereby preventing *C/EBP-alpha* and *PPAR-gamma* activation and blocking hMSC adipogenesis (Figure 4).[34,35]

Furthermore, GW9662 inhibition of *PPAR-gamma* led to a significant reduction of hMSC differentiation (even though this was partial) (Figure 4(a) and (b)). Similar results were observed when assessing *PPAR-gamma* gene expression in the presence of GW9662 (Figure 3(b)). These results could have been due to several factors. The literature has reported that regulation of transcription factors, *PPAR-gamma* type (nuclear hormone receptor) occurs mainly through ligand binding, and these induce or inhibit these transcription factors' transcriptional activity.[36] Although there is also evidence that other mechanisms may modulate *PPAR-gamma* activation, repression, and intracellular distribution, such as the action of co-activators and/or phosphorylation, which are independent of ligand binding.[37–39] Some of these mechanisms may thus account for the partial inhibition of the *PPAR-gamma* pathway by GW9662. Furthermore, it has been described that *PPAR-gamma* has elements of response to other transcription factors, including *C/EBP-alpha*, which would become active at the moment of inhibiting the *PPAR-gamma* pathway by exerting positive control over this transcription factor,[40] resulting in partial inhibition of *PPAR-gamma* expression and adipogenic differentiation.

The GW9662 inhibitor concentration used was insufficient to completely block the *PPAR-gamma* pathway. Considering that GW9662 acts as a competitive inhibitor and its half-life is about 2 h and rosiglitazone is about 20 h.[41] Potential inhibition at different GW9662 concentrations increased by 5–10 times above the rosiglitazone concentration was evaluated; the results showed that at 10 times higher inhibitor concentration, D&R-induced adipogenic differentiation became completely suppressed, while inhibition was 99% at a fivefold higher concentration (Supplementary Figure S3A and B). Regarding *PPAR-gamma* gene expression, it was observed that both concentrations significantly inhibited this transcription factor's expression levels (Supplementary Figure S4); concentrations greater than 50 μM GW9662 were harmful for cells. These results demonstrated that the rosiglitazone and GW9662 concentration ratio was decisive in terms of obtaining efficient inhibition of the *PPAR-gamma* pathway. A 10 μM rosiglitazone concentration was used here, given that this concentration led to obtaining the highest percentage of hMSC adipogenic differentiation (Supplementary Figure S5A and B). These results were consistent with Wang's work which showed that the adipogenic effect of rosiglitazone was maximized at doses of 10 and 30 μM, in D1 cells a multipotential cell line derived from mouse bone marrow.[21] Whereas 10 μM GW9662 was determined according to inhibitor specificity since it has been reported that higher than 10 μM concentrations of this inhibitor may act as agonists for other signaling pathways.[25]

As no changes were observed in *PPAR-gamma* gene expression levels at 10 μM GW9662, we asked ourselves whether GW9662 (at this concentration) would regulate this gene's transcriptional activity. This led to evaluating the expression of target genes (i.e. *aP2*, *LPL*, PEPKC, and *adiponectin*) forming part of a mature adipocyte's gene profile.[1,3,42,43] The results showed a significant inhibition of the expression of all target genes tested 14 days after beginning adipogenic activation with D&R (Supplementary Figure 2A). Although GW9662 was insufficient to completely inhibit the *PPAR-gamma* pathway, the results demonstrated a direct interaction between the *C/EBP-alpha* and *PPAR-gamma* pathways and that both pathways were crucial for inducing hMSC adipogenesis.

Regarding the maturation stage, it was found that adipocytes produced from D&R-exposed hMSC had the gene profile characteristic of functional adipocytes (Figure 5); mRNA abundance was lower in D&R-exposed hMSC than in those exposed to CC at all the times studied here. This was an unexpected result because the percentage of Nile Red-stained cells was lower when hMSCs were triggered by D&R rather than CC (Figure 1(b)); nonetheless,

there was no difference for *adiponectin* mRNA and the opposite was observed for *leptin* mRNA (Figure 5). As adipokine expression is a marker of final maturation, these mRNA level differences might be attributed to adipocyte functionality. Accordingly, adipocytes produced from D&R-exposed hMSC had no proliferation potential (Figure 6) and glucose uptake was dose dependent on insulin stimulus (Figure 7). These results agreed with properties in terms of function and maturation described for adipocytes *in vivo* in normal conditions, while undifferentiated cells and adipocytes produced from CC-exposed hMSC did not share these characteristics.

The pertinent literature has reported that an ability to incorporate glucose regarding insulin is an exclusive property of adipocytes which have completed their maturation and which would be in the final stages of adipogenesis.[44–46] The foregoing and our results thus suggested that adipocytes produced by CC exposure would be immature adipocytes which had not acquired glucose transport mechanisms and dysfunctional adipocytes given an alteration in the same mechanism. The ultimate test of mature adipocyte functionality would be its ability to secrete adipokines. While adipocytes produced from CC-exposed hMSC marginally secreted adiponectin and did not secrete leptin, those produced with D&R abundantly secreted both adipokines (Figure 8). Such differences could be attributed to the different degrees of cell adipogenic differentiation; adipocytes produced with CC were partially differentiated (immature), therefore lacking the machinery to induce leptin expression and secretion (Figures 5 and 8). Regarding adipocyte functionality, the literature has reported that beta adrenergic agonists, lipolytic hormones (ACTH and thyrotropin-stimulating hormone), catecholamines, and analogs of camp (caffeine, theophyl-line, IBMX) interfere with both adipokines' synthesis and secretion.[47,48] The low level of leptin secreted by adipocytes differentiated with CC may thus have been due to the presence of IBMX producing dysfunctional adipocytes.

It has usually been considered that neutral fat droplet accumulation in a cell's cytoplasm is indicative of terminal adipogenic differentiation. However, it has been shown that cells having such morphology fail to express mature adipocyte genes, proliferate, and can become dedifferentiated.[49] A combined analysis was thus used which included morphological, genetic, and functional parameters to assess the degree of maturation. A minimum stimulus was defined which would induce adipogenesis from undifferentiated MSC. Compared to CC, D&R has several advantages; it is simpler, induces the differentiation of MSC from several mammalian species including those refractory to CC, and allows functional adipocytes to be produced from hMSC. This new adipogenic stimulus should be a useful tool for MSC characterization, studying adipogenesis pathways and producing functional adipocytes which might be used in cell therapy, for example in plastic and reconstructive surgery.

Authors' contributions: DC conceived and carried out the experiments and analyzed the data, FE, ME, MA-R, and CP carried out the experiments; LS analyzed the data; and PC conceived the study, analyzed, and interpreted data. All the authors were involved in writing the paper and gave their final approval to the first and revised versions.

CONFLICT OF INTEREST

DC and PC are the inventors concerned in the aforementioned patent application filed by the Universidad del Desarrollo.

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