MCAM knockdown impairs PPARγ expression and 3T3-L1 fibroblasts differentiation to adipocytes

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Abstract

We investigated for the first time the expression of MCAM (Melanoma cell adhesion molecule) and its involvement in the differentiation of 3T3-L1 fibroblasts to adipocytes. We found that MCAM mRNA increased subsequent to the activation of the master regulator of adipogenesis, PPARγ, and this increase was maintained in the mature adipocytes. On the other hand, MCAM knockdown impaired differentiation and induction of PPARγ as well as expression of genes activated by PPARγ. However, events that precede and are necessary for early PPARγ activation such as C/EBPβ induction, β-catenin downregulation and ERK activation, were not affected in the MCAM knockdown cells. In keeping with this, the increase in PPARγ mRNA that precedes MCAM induction was not altered in the knockdown cells. In conclusion, our findings suggest that MCAM is a gene upregulated and involved in maintaining PPARγ induction in the late but not in the early stages of 3T3-L1 fibroblasts adipogenesis.

Keywords

MCAM/CD146; 3T3-L1 fibroblasts; adipogenesis; PPAR gamma; C/EBP beta

INTRODUCTION

Obesity is a frequent health problem due to an increase in the number (hyperplasia) and size (hypertrophy) of adipocytes which is associated with metabolic syndrome, type 2 diabetes mellitus, hypertension and cardiovascular disease [1, 2]. Adipocytes are derived from mesenchymal stromal cells, these are precursor cells which are able to differentiate to

several lineages such as: osteocytes, chondrocytes and adipocytes. The knowledge of the signaling involved in the development of adipocytes may help in the control of obesity and also may be useful to understand how to select a specific cell fate in the case of tissue regeneration therapy [3].

Adipogenesis is a coordinated and complex process that starts with the upregulation of two early transcription factors CCAAT/enhancer-binding protein beta (C/EBPβ) and C/EBPδ and then they induce the expression of Peroxisome proliferator-activated receptor gamma (PPARγ), the master gene in adipogenesis which, in turn, increases the expression of proteins associated with the adipocyte phenotype [4]. Accordingly, a dominant-negative PPARγ mutant inhibits adipogenesis [5, 6].

3T3-L1 fibroblasts are precursor cells used as a model to study adipogenesis. These preadipocytes are induced to differentiate with a mixture containing insulin, dexamethasone and methylisobutylxanthine. A few days later, cells become round and start to accumulate lipid droplets and terminal differentiation is attained 7 to 10 days after induction.

In order to identify genes relevant for adipogenesis, we have previously performed a microarray analysis of genes regulated during differentiation of 3T3-L1 fibroblasts [7]. In this microarray, among the genes that were upregulated, we found genes known to be involved in adipogenesis and also identified the *Mcam* gene. We decided to analyze MCAM as little is known about its regulation and role during adipocyte differentiation.

MCAM (melanoma cell adhesion molecule) also known as CD146, is an integral membrane protein belonging to immunoglobulin (Ig) superfamily of cell adhesion molecules. It was originally identified as a marker associated to tumor progression and metastasis in human melanoma, but later was found to be present in other malignant lesions as well as in normal tissues [8, 9]. MCAM is also a mesenchymal stromal cell (MSC) marker whose expression positively correlates with the differentiation potential [10]. The protein structure is composed of an extracellular region with five Ig-like domains (V-V-C2-C2-C2), a transmembrane region and a cytoplasmic domain and it is conserved among human, mouse and other organisms [9]. MCAM functions as a cell surface receptor that signals into the cell upon binding of different ligands. Laminin-411, Wnt5a, netrin-1 has been described as ligands for MCAM [11–13]. In addition, MCAM has been proposed as a co-receptor for VEGF-R2 and is involved in VEGF signaling through AKT/p38 MAPKs/NF-κB to promote cell migration and angiogenesis in endothelial cells [14]. It has also been shown that upon its engagement in endothelial cells, MCAM recruits and activates Fyn kinase leading to a protein kinase cascade that has been proposed to be linked to actin cytoskeleton remodeling [15].

In this paper, we investigate the expression of MCAM and its involvement in 3T3-L1 fibroblasts differentiation to adipocytes.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM), antibiotics, trypsin, insulin, Bodipy 493/503 and Lipofectamine 2000 were obtained from Invitrogen (Carlsbad, CA, USA). Methylisobutylxanthine, dexamethasone were obtained from Sigma-Aldrich (St. Louis, MO, USA). Puromycin was purchased from Invivogen (San Diego, CA, USA). 3T3-L1 fibroblasts were obtained from Asociación Banco Argentino de Células (origin: ATCC), NIH 3T3 from ATCC and HEK293FT from Invitrogen. 3T3-L1 cells could not be authenticated due to the lack of an STR DNA profile database for mouse cells. However, the adipocyte phenotype that we observed only after differentiation is consistent with the fact that the cells used were indeed 3T3-L1 fibroblasts. Fetal Bovine Serum (FBS) was purchased from Natocor (Argentina).

Cell culture and differentiation induction

3T3-L1 and NIH 3T3 fibroblasts were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 0.1 mg/mL streptomycin, 100 U/mL penicillin and 250 ng/mL fungizone (DMEM + 10% FBS). In differentiation experiments, plated cells were allowed to reach confluence and two days later differentiation was induced with a mixture containing: 10 μg/mL insulin (I), 0.1 μM dexamethasone (D), and 0.5 mM methylisobutylxanthine (M) in DMEM with 10% FBS (IDM). After 3 days medium was replaced with DMEM + 10% FBS supplemented with 10 μg/mL insulin and cells were fed every 2 days thereafter with DMEM + 10% FBS.

In some experiments, 2-day postconfluent 3T3-L1 fibroblasts were stimulated with individual inducers (I, D, M) or combinations of two inducers (ID, DM, IM) at the concentrations indicated above. After 3 days culture medium was replaced with DMEM + 10% FBS alone or supplemented with 10 μg/mL insulin in those assays induced in the presence of insulin.

Construction of lentiviral vectors, virus production and transduction of 3T3-L1 fibroblasts

A fragment coding a small hairpin RNA (shRNA) that targeted murine *Mcam* was subcloned into the pLKO.1 plasmid [16] according to the protocol available from Addgene (http:// www.addgene.org/tools/protocols/plko/). The shRNA sequence 5'-CCGACTCGTAAGAGTGAATTT-3', which targets the coding DNA sequence (CDS) of the murine *Mcam* mRNA, was selected from the TRC RNAi Consortium library (http:// www.broad.mit.edu/genome_bio/trc/rnai.html), clone ID TRCN0000113082. The empty pLKO.1 vector, without the shRNA construct and the stuffer sequence, was used as a negative control.

For lentivirus production, exponentially growing HEK293FT cells cultured in 6-well plates were transfected in 1.5 ml of medium using 6 μl of Lipofectamine 2000 with 1.2 μg of either the shRNA containing vector or the empty vector, in combination with 1 μg of the pCMV-R8.9 packaging plasmid and 0.3 μg of the pCMV-VSVG envelope plasmid. Media was

replaced with fresh one after 6 hours. Lentivirus containing media were harvested at 48 h and 72 h after transfection and filtered through a 0.45 μm filter.

To establish cell lines stably expressing shRNA or control vector, exponentially growing 3T3-L1 fibroblasts in 6-well plates were transduced with virus-containing media supplemented with 8 μg/mL polybrene. After 24 h, 3T3-L1 cells were transduced again with lentivirus containing media for another 24 h. Then, transduced cells were subjected to selection with culture media supplemented with 5 μg/mL puromycin for 4–6 days.

Oil-Red-O staining

3T3-L1 adipocytes were washed three times with phosphate-buffered saline (PBS) and then fixed for 30 min with 4% formaldehyde in PBS. Three volumes of Oil-Red-O (0.4% in isopropanol) were diluted with two volumes of water, filtered, and added to the fixed cell monolayers for 30 min at room temperature. Cells were then washed with water and the stained lipid droplets were visualized by light microscopy and photographed.

Western-blots

3T3-L1 cells grown in 6-well plates were harvested with a cell scraper in PBS 24 h after induction of differentiation for C/EBPβ or 72 h for β-catenin, vortexed and before the addition of Laemmli sample buffer [17] an aliquot was obtained for protein quantification using the method of Bradford with serum albumin as standard [18]. For ERK determination, two-day postconfluent 3T3-L1 cells were treated for 1 h with DMEM + 1 % FBS then cells were induced to differentiate for 30 min. After the treatments, cells were harvested in Laemmli sample buffer and vortexed. Samples were loaded in SDS-polyacrylamide gels, electrophoresed and transferred to nitrocellulose membranes. After blocking with 5% non-fat dry milk for 1 h, membranes were incubated overnight at 4 °C with primary antibody at a 1:1000 dilution, and then with HRP-conjugated secondary antibody at a 1:1000 dilution for 1 h at room temperature. Detection was performed with a chemiluminescent reagent. GAPDH was used as the loading control. C/EBPβ (C-19, cat # sc-150), GAPDH (6C5, cat # sc-32233), p-ERK (E-4, cat # sc-7383), ERK 2 (C-14, cat # sc-154) antibodies were from Santa Cruz (Santa Cruz Biotechnology, CA, USA) and β-catenin (D10A8, cat# 8480) was from Cell Signaling Technology (Danvers, MA, USA). Secondary antibodies used, anti-mouse IgG-HRP (C2011, cat# sc-2005) and anti-rabbit IgG-HRP (cat# sc-2004), were both from Santa Cruz.

Immunofluorescence microscopy

3T3-L1 cells were cultured on glass coverslips. 7 days after induction of differentiation cells were fixed for 15 min with 4% formaldehyde in PBS at room temperature, permeabilized with 0.5% saponin in PBS for 10 min and blocked with 1% BSA – 0.3 M glycine in PBS for 1 h at room temperature. Cells were incubated with 1:75 dilution of anti-MCAM primary antibody (EPR 3208, cat # ab75769, Abcam, Cambridge, MA, USA) or 1:100 anti-PPARγ (C26H12, cat# 2435, Cell Signaling Technology, Danvers, MA) for 1 h at 37 °C in a humidified chamber, and then incubated for 1 h with 1:500 dilution of goat anti-rabbit Alexa 555-conjugated secondary antibody (cat# A21428, Invitrogen). Finally, nuclei were stained with Hoechst 33258 and, when indicated, lipid droplets with Bodipy 493/503. Coverslips

were mounted on slides and cells were visualized and photographed in an Olympus IX-71 inverted microscope equipped with a QImaging EXi Aqua monochrome camera. In any given experiment all images were acquired using the same settings and 8–10 random fields were photographed from each sample. Images were processed and analyzed using Fiji software [19]. Background correction was performed by using the Rolling Ball algorithm for Hoechst images or by substracting the mean intensity value from a region without cells for MCAM and PPARγ images. Blue, green or red lookup tables (LUTs) were used to colour grayscale images. Then, for better visualization, minor adjustements to maximum intensity were made to the whole figure. For cell counting studies, the number of Hoechst-stained nuclei and lipid droplets-containing cells in multiple fields were determined. At least 1,000 nuclei were scored in each replicate.

RNA extraction and RT-qPCR analysis

Total RNA purification from 3T3-L1 fibroblasts was performed with TRI Reagent (Molecular Research Center) according to the manufacturer's instructions, pellets were dissolved in nuclease-free water and RNA concentration was determined with NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). 2 μg of RNA and 0.5 μg of Oligo $(dT)_{15}$ were used for reverse transcription with RevertAid Reverse Transcriptase (Thermo Fisher Scientific). mRNA relative expression was determined by quantitative real-time PCR using a Bio-Rad MyIQ2 thermal cycler. Each PCR reaction was performed using Platinum Taq Polymerase (Invitrogen) in a final volume of 25 μL, containing 5 μL of a 1:10 dilution of first-strand cDNA and 5 pmol of each primer. The following cycling conditions were used: 94°C for 5 min, followed by 40 cycles of 94°C for 20 s, 58°C for 30 s and 72°C for 40 s. Target gene mRNA expression was normalized to acidic ribosomal protein (*Rplp0*) as a reference gene. MCAM and Perilipin 1 (*Plin1*) primers were designed using Primer3 v0.4.0 software [20], *Rplp0*, Peroxisome proliferator activated receptor gamma isoform 2 (*PPARγ2*), and Adiponectin (*Adipoq*) primer sequences were obtained from published reports [21–23]. The sequences of primers used are shown in Table 1.

Statistical analysis

The experiments were carried out three times unless otherwise stated. All data are expressed as mean ± SD. Statistical analysis was performed using GraphPad Prism 6 software. More than two samples were compared by One-Way ANOVA followed by Bonferroni *post hoc* test and two samples by Student's t-test, p values below 0.05 were considered significant.

RESULTS

Effect of the inducers of differentiation on MCAM upregulation in 3T3-L1 fibroblasts

In the present investigation, we first analyzed the dependence of MCAM upregulation with the inducers of the mixture used to differentiate 3T3-L1 fibroblasts to adipocytes. In accordance with our previous microarray data, we found that MCAM mRNA level was increased in 3T3-L1 fibroblasts treated with differentiation mixture (IDM). However, treatment with any of the inducers alone failed to stimulate MCAM expression (Fig. 1A). Furthermore, none of the inducers alone was able to induce adipogenesis (Fig. 1B). When 3T3-L1 fibroblasts were treated using different combinations of inducers, only in the case of

DM, differentiation was attained although in a lower extent than when the three inducers were used. This is in agreement with previous reports [24, 25]. Interestingly, MCAM was only upregulated by DM and neither ID nor IM were able to induce MCAM or differentiation (Fig. 1).

The increase of MCAM after induction of differentiation of 3T3-L1 fibroblasts was confirmed by immunofluorescence. In keeping with the results shown in Figure 1, we found an increase in the amount of this protein in the differentiated 3T3-L1 cells which were also stained with Bodipy 493/503 to visualize accumulation of cytosolic lipids (Fig. 2). As previously reported, we observed that this protein is localized at the plasma membrane (9).

Time-course of MCAM induction

We also evaluated the time-course of MCAM induction and found that MCAM mRNA continuously increased from day 3 after the addition of IDM and the increase in expression was maintained in mature adipocytes. In contrast, the mRNA of PPARγ, the master gene of adipogenesis, was increased after 24 h and Perilipin 1, a gene which is known to be upregulated by PPARγ [26, 27] after 48 h (Fig. 3).

Dependence of MCAM induction on adipogenesis

To further investigate the importance of differentiation to adipocytes in the induction of MCAM, we used NIH 3T3 fibroblasts as a cell model that do not differentiate to adipocytes after the addition of the differentiation mixture. After treatment of NIH 3T3 cells with IDM, we did not find lipid containing cells that resembled adipocytes neither did we find a significant increase in Perilipin 1 or MCAM mRNA expression as we observed in 3T3-L1 cells (Fig. 1A). These results support the importance of adipogenesis in the induction of MCAM.

Effect of MCAM knockdown on the differentiation of 3T3-L1 fibroblasts to adipocytes

To characterize the role of MCAM during 3T3-L1 fibroblasts differentiation we generated 3T3-L1 cells that stably expressed a shRNA against MCAM mRNA. We analyzed MCAM mRNA levels in knockdown cells (MCAM-KD) and found that MCAM mRNA upregulation was reduced on day 4 and 7 after induction of differentiation compared to control cells (Fig. 4A). When we evaluated the ability of these cells to differentiate, we found that after induction of differentiation the cytosolic accumulation of lipids which was evaluated by Oil-Red-O staining was inhibited in the MCAM-KD cells (Fig. 4B). In keeping with this, as shown in Fig. 5A, we also found impairment of MCAM at the protein level in MCAM-KD cells which was evaluated by immunofluorescence. When we counted under the microscope the number of adipocytes relative to the number of Hoechst-stained cells, we consistently observed a significant decrease in the amount of adipocytes in knockdown cells (0.33±0.09 vs. 0.09±0.02, p<0.01, n=7).

Then, the effects of MCAM knockdown on the amount of PPARγ, Perilipin 1 and Adiponectin mRNA was evaluated in the MCAM-KD cells with respect to those treated with the empty vector. Consistent with the inhibition of the adipogenesis there was a decrease in PPARγ mRNA, as well as in the mRNA expression of Perilipin 1 and Adiponectin, which

are both upregulated by PPARγ (Fig. 4A). The decrease of nuclear PPARγ was also confirmed by immunofluorescence (Fig. 5B).

To further investigate the effect of MCAM knockdown on adipogenesis, we analyzed two proteins which are regulated in early adipogenesis and are involved in PPARγ upregulation [28, 29]: C/EBPβ, a transcription factor upregulated in the first 24 h [4] and β-catenin, a protein involved in canonic Wnt signaling, which is known to be decreased in adipogenesis.

First, we analyzed the amount of C/EBPβ in MCAM-KD cells treated with or without differentiation mixture and compared the increase in the amount of this protein after induction of differentiation with that obtained in cells transduced with an empty vector. We found that 24 h after induction of differentiation the expected increase in C/EBPβ was still present in MCAM-KD cells (Fig. 6A).

On the other hand, in keeping with previous reports, it was found that in control cells induction of differentiation provoked a decrease in the content of β-catenin and this decrease was not affected in MCAM-KD cells (Fig. 6B).

Finally, we also found that another early event involved in this differentiation process, the activation of a MAP kinase, ERK, which takes place in the first hours after the addition of the differentiation mixture [30], was maintained in the MCAM-KD cells in spite of the inhibition in differentiation (Fig. 6C).

Since our results suggest that the early events in adipogenesis that precedes and are necessary for PPARγ activation were not impaired in MCAM-KD cells, we analyzed if the early increase of PPARγ mRNA at 24 h post differentiation induction (as shown in figure 3) was affected in MCAM-KD cells. On the contrary, we found that PPARγ expression was only impaired 3 days after the induction of differentiation in MCAM-KD cells when MCAM downregulation was also evident (Fig. 6D).

4. DISCUSSION

In the present work, we demonstrated for the first time that MCAM expression was increased during differentiation of 3T3-L1 fibroblasts and it remained highly expressed in 3T3-L1 adipocytes. Consistently, treatment of cells with either one inducer or combinations thereof which were unable to trigger differentiation, could not upregulate MCAM. In addition, only the induction with a mixture containing DM was able to induce MCAM expression and adipogenesis although to a lesser extent than the complete differentiation mixture (IDM). Furthermore, treatment of non-adipogenic NIH 3T3 cells with the complete differentiation mixture neither induced MCAM nor Perilipin 1, which is a protein present in lipid droplets and is involved in cAMP-mediated lipolysis in adipocytes [31].

We also found that PPARγ was induced in 3T3-L1 cells 24 h after the addition of the differentiation mixture, which is in keeping with previous results [21, 28, 32], while Perilipin 1, a gene regulated by PPARγ, and MCAM were induced later in the adipogenic program. Taking this into account and the fact that the induction of MCAM is associated with adipogenesis, it is likely that MCAM is another gene regulated by PPARγ. In keeping

with this we have seen that an activator of PPARγ, piogllitazone, is able to enhance MCAM induction and, on the contrary, TNFα, an inhibitor of PPARγ expression, impairs MCAM induction (unpublished observation). Whether this is a direct or indirect effect of PPARγ on MCAM gene expression remains to be determined.

To investigate the importance of MCAM induction in adipogenesis, we analyzed the effect of the inhibition of MCAM expression in 3T3-L1 fibroblasts differentiation. We found that in MCAM knockdown cells differentiation to adipocytes was impaired. Interestingly a previous report showed that downregulation of MCAM in human bone marrow multipotent mesenchymal stromal cells (hMSC) reduced the potential of these cells to differentiate into adipogenic and osteogenic lineages [33].

The impaired adipogenesis in MCAM-KD cells was associated with a decrease in the induction of PPARγ as well as genes upregulated by this transcription factor and important for adipocyte function such as Perilipin 1 and Adiponectin. Adiponectin is an adipokine which has been found to be associated with increased insulin sensitivity and has been shown to be reduced in insulin resistant patients [34, 35]. The positive contribution of MCAM to adipogenesis and adipokine expression is in agreement with a recent report [3]. In this report adipose tissue-derived stem cells were isolated from the stromal vascular fraction of human abdominal fat and MCAM $^+$ cells were selected and compared to MCAM$^-$ cells. The presence of MCAM favor adipogenesis and differentiated MCAM $^+$ cells expressed higher levels of two adipocyte markers Adiponectin and Leptin.

On the other hand, early events in the differentiation process which have been involved in promoting the enhancement of PPARγ expression such as the increase in the amount of C/EBPβ and in ERK phosphorylation as well as the downregulation of Wnt-β-catenin signaling [4, 29, 30], were maintained in the knockdown cells. Thus, MCAM upregulation does not seem to be required for any of them. According to our results MCAM was not involved in PPARγ upregulation at the early stages of adipogenesis which is in keeping with the increase of MCAM mRNA after that of PPARγ.

PPARγ is known to be the master transcription factor in adipogenesis and in adipocytes since it is involved in the expression of proteins important in adipocyte function like C/EBPα, which is upregulated by PPARγ and has been involved in a positive loop to keep PPARγ expression in late adipogenesis and adipocytes [6]. Thus, the decrease in gene expression found in the knockdown cells (PPARγ, Perilipin 1, Adiponectin), may be due to the involvement of MCAM in the mechanisms that maintain PPARγ expression in late adipogenesis. Consistently, the reduced expression of MCAM in the knockdown cells did not impair the increase in PPARγ mRNA that was found 24 h after induction of adipogenesis. Similarly to MCAM, Lipin 1 is another protein that has been reported to be expressed late in adipogenesis, subsequent to PPARγ activation and its expression is also maintained in adipocytes. In agreement with our findings, in Lipin 1 knockdown cells adipogenesis and PPARγ induction are inhibited but the early stages of adipocyte differentiation are not affected. It has been shown that Lipin1 acts as a transcription regulator which is induced by C/EBPα and contributes to PPARγ activation and therefore reinforces the positive loop between C/EBPα and PPARγ in 3T3-L1 fibroblasts [21]. In

contrast, MCAM has not been described as a transcription factor but it has been involved in regulation of gene expression through different signaling pathways [36, 37]. However, the outside-in signaling pathway mediating MCAM regulation of cellular transcription are poorly understood so far. More studies are necessary to elucidate the events involved in MCAM signaling from the cell surface that contributes to maintain the induction of PPARγ and other adipocytes specific genes. Interestingly, among the different proposed ligands of MCAM, laminin 411 and galectin-1, both seem to favor adipogenesis. In the case of laminin 411 (α4,β1,γ1) it has been reported that during 3T3-L1 fibroblasts differentiation to adipocytes its expression is increased from day 0 to day 6 [38]. In addition mice with a null mutation of the laminin α4 gene exhibited reduced weight gain in response to both age and high fat diet. The mass of epididymal adipose tissue was specifically decreased in the knock-out mice, and there was also impaired lipogenesis in this adipose tissue [39]. On the other hand, two recent reports [40,41] have shown the involvement of galectin-1 in stimulation of adipogenesis. It was found that thiodigalactoside, which is an inhibitor of galectin-1, reduced fat accumulation in cultured adipocytes and that *in vivo* administration to Sprague Dawley rats, inhibited high fat diet-induced body weight gain by inhibiting adipogenesis and lipogenesis and increasing thermogenesis. Moreover, knockdown of Gal-1 in 3T3-L1 cells, attenuated adipogenesis and lipogenesis [40]. In addition, Lactulose another inhibitor of galectin-1, reduced adipogenesis by downregulation of PPARγ and C/EBPα in 3T3-L1 cells and *in vivo* administration alleviated high fat diet induced body weight gain [41]. Thus, it is possible that these stimulatory effects of laminin 411 and/or galectin-1 in adipogenesis are mediated by interaction with MCAM. Further studies are necessary to evaluate this possibility.

In conclusion, our findings indicate that MCAM expression is increased three days after induction of differentiation of 3T3-L1 fibroblasts and is necessary to keep PPARγ induction in the late stages of adipogenesis in 3T3-L1 fibroblasts as well as in the adipocyte.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

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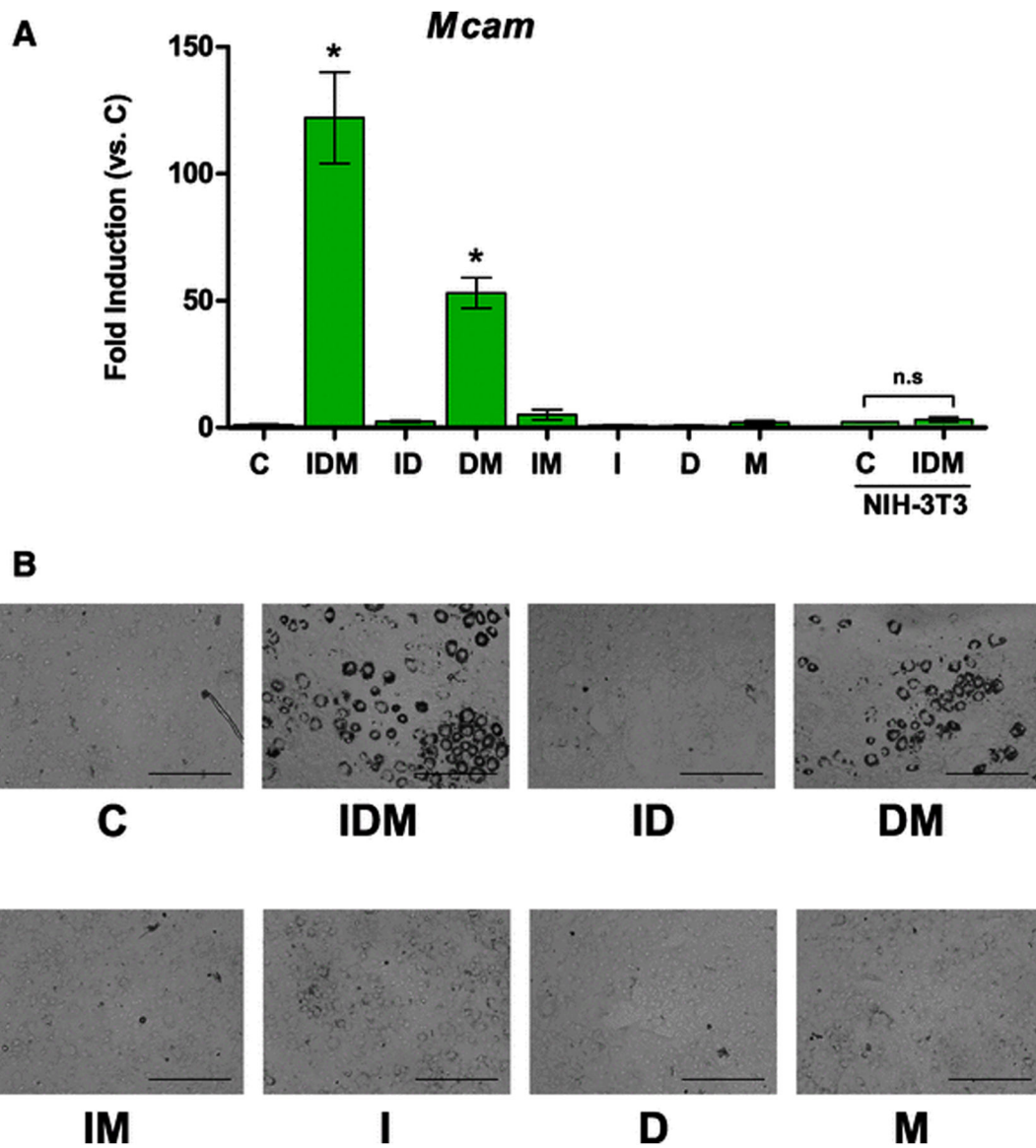
Figure 1. Effect of the inducers of differentiation on MCAM expression.
Two-day postconfluent 3T3-L1 fibroblasts were treated with DMEM+10% FBS alone (control, C), or with the addition of insulin (I), dexamethasone (D) or methylisobutylxanthine (M) alone, or with different combinations of these inducers. Postconfluent NIH 3T3 cells were treated with DMEM+10% FBS alone (control, C) or with IDM. (**A**) After seven days, *Mcam* mRNA levels were analyzed by RT-qPCR and its expression was normalized to *Rplp0* mRNA. Data are expressed as mean ± SD relative to control treatment of duplicates from a representative experiment repeated three times with similar results. * $p<0.05$ vs. C 3T3-L1, Bonferroni *post hoc* test and n.s., not significant, Student's t test. (**B**) To evaluate the extent of differentiation in 3T3-L1 cells, cytosolic lipid droplets were stained with Oil-Red-O. Scale bar = 300 μm.
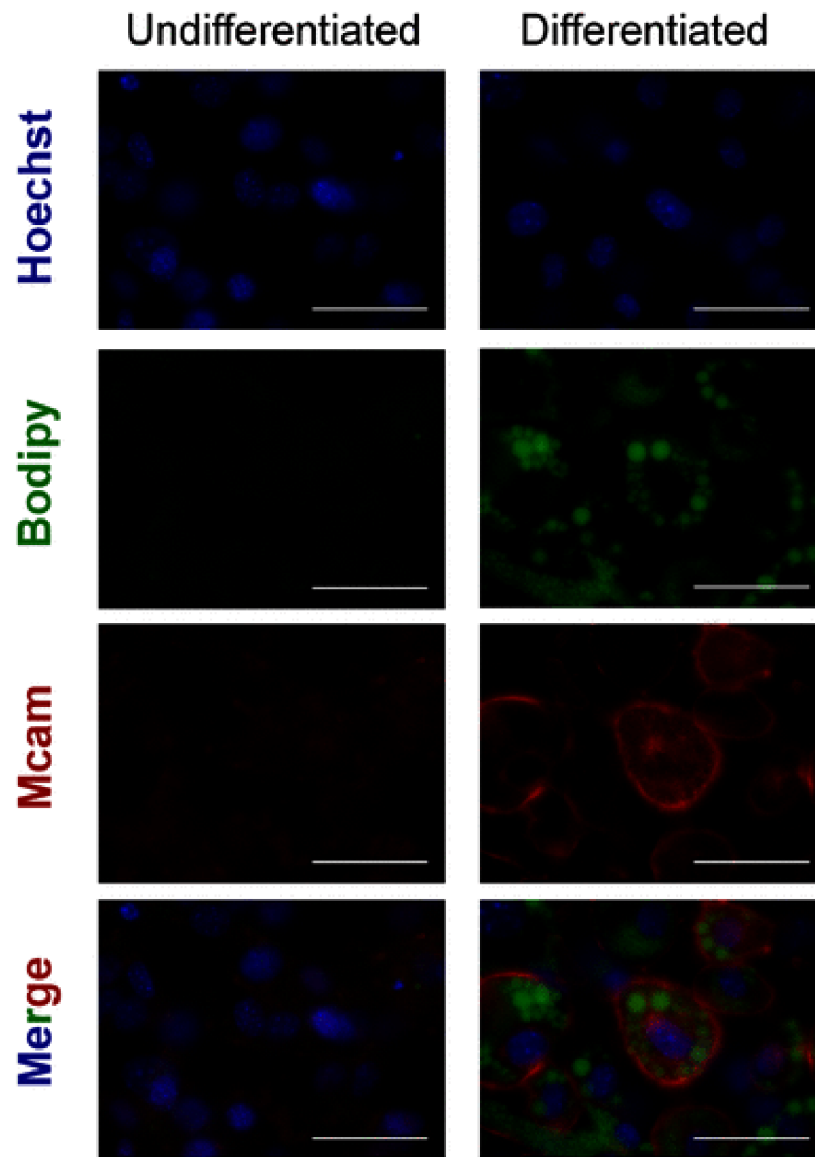
Figure 2. Immunofluorescence detection of MCAM in differentiated 3T3-L1 cells.
Two-day postconfluent 3T3-L1cells were induced to differentiate and seven days later the
amount of Mcam was analyzed by immunofluorescence (red), as described in Matherials
and Methods section. Uninduced cells are shown as control (Undifferentiated). Nuclei were
stained with Hoechst 33258 (blue) and lipid droplets were stained with Bodipy 493/503
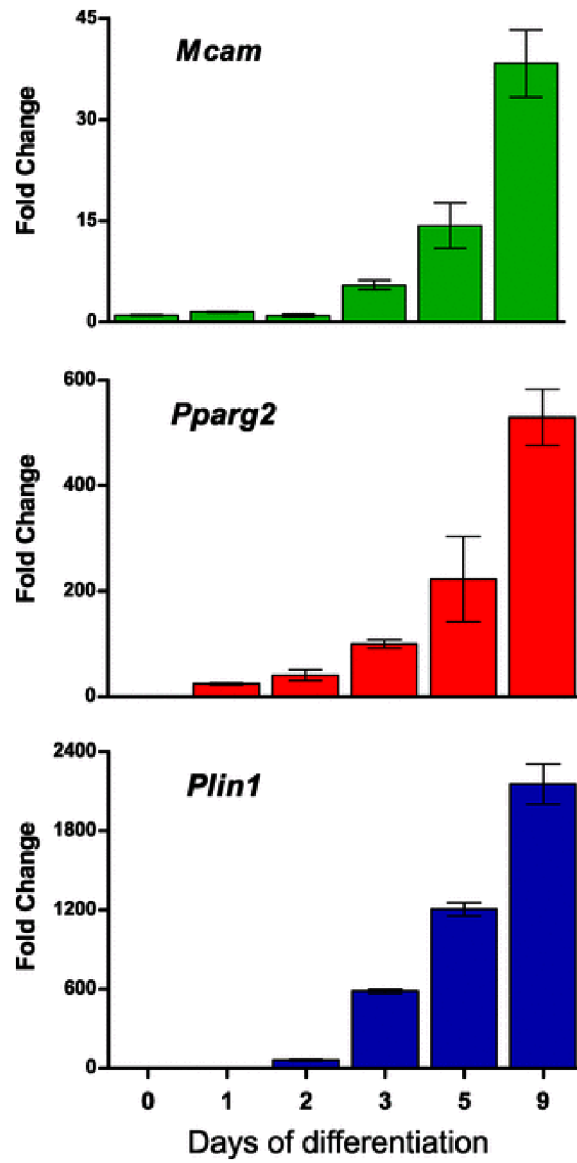(green). Scale bar = 50 μm.

Figure 3. Analysis of MCAM, PPARγ and Perilipin 1 mRNA expression during differentiation of 3T3-L1 fibroblasts to adipocytes.

Two-day postconfluent 3T3-L1 fibroblasts were induced to differentiate and the levels of *Mcam*, *PPARγ2* and *Plin1* mRNAs were analyzed at different time points after induction by RT-qPCR, as described in Materials and Methods. Expression levels of each mRNA were normalized to *Rplp0* mRNA. For each gene data are shown as mean ± SD relative to the levels at day 0 of duplicates from a representative experiment repeated three times with similar results.
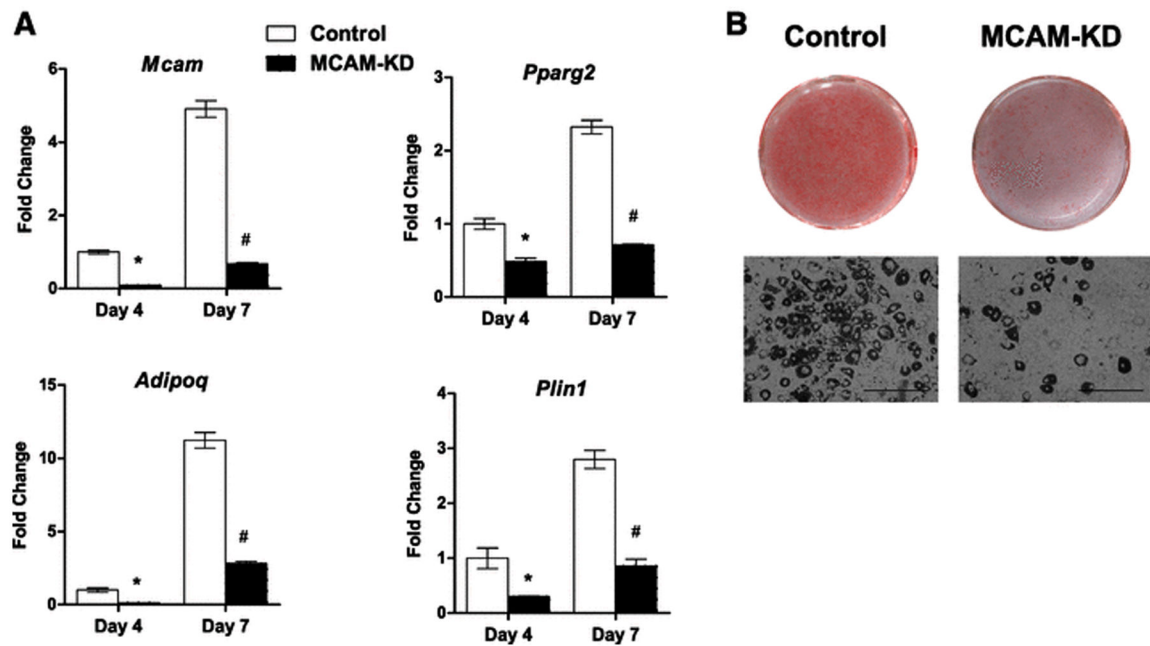
Figure 4. Effect of MCAM knockdown on the differentiation of 3T3-L1 fibroblasts to adipocytes. 3T3-L1 fibroblasts were stably transduced with lentivirus containing an anti-Mcam shRNA (MCAM-KD) or an empty vector (Control) and two days after reaching confluence were induced to differentiate. (**A**) *Mcam*, *PPARγ2, Adipoq* and *Plin1* mRNA levels were analyzed by RT-qPCR at days 4 and 7 after induction. Each gene mRNA expression was normalized to *Rplp0* mRNA. RT-qPCR data are expressed as mean ± SD relative to Control cells at day 4 of duplicates from a representative experiment repeated three times with similar results. * p<0.05 vs. Control cells at day 4, # p<0.05 vs. Control cells at day 7 (Student's t-test). (**B**) On day 7 of adipogenesis cells were stained with Oil-Red-O and photographed. Scale bar = 300 μm.
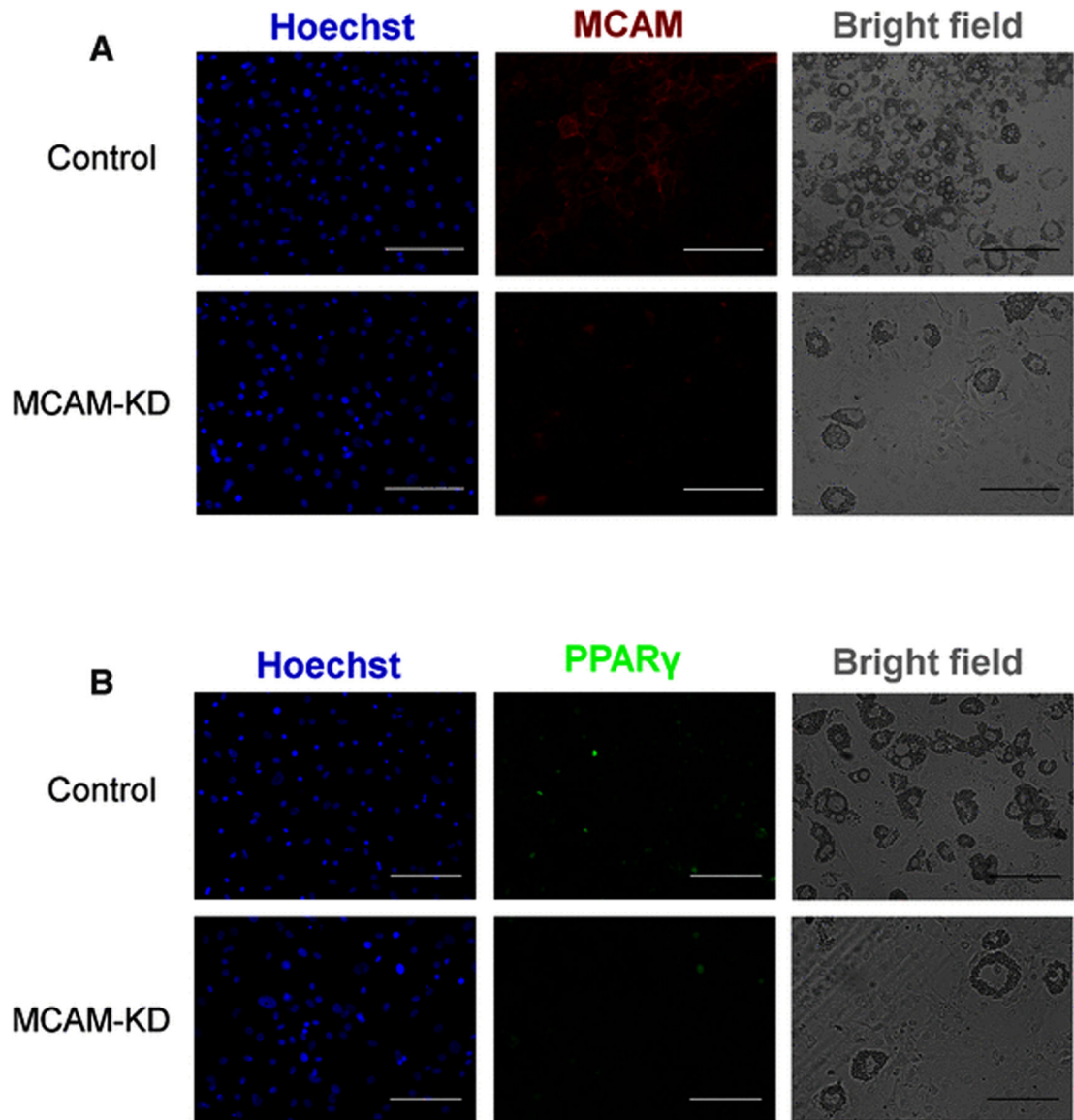
Figure 5. Immunofluorescence detection of MCAM and PPARγ in differentiated MCAM-KD 3T3-L1 cells.
3T3-L1 fibroblasts were stably transduced with lentivirus containing an anti-MCAM shRNA (MCAM-KD) or an empty vector (Control) and two days after reaching confluence were induced to differentiate. Seven days later the amount of MCAM (**A**) or PPARγ (**B**) was analyzed by immunofluorescence, as described in Materials and Methods section. Nuclei stained with Hoechst 33258 (blue) and adipocytes (bright field) are also shown. Scale bar = 150 μm.
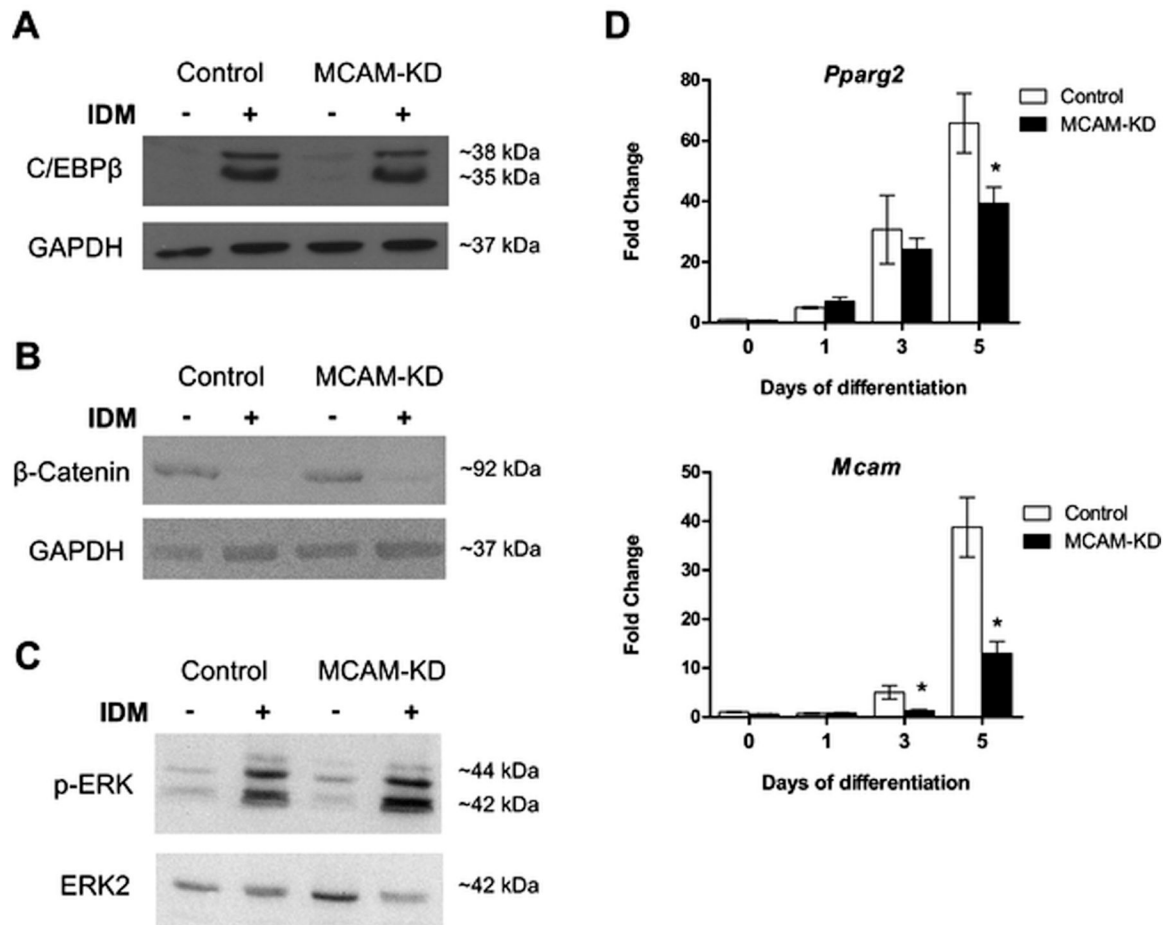
Figure 6. Effect of MCAM knockdown on early events of 3T3-L1 fibroblasts differentiation to adipocytes.

3T3-L1 fibroblasts were stably transduced with lentivirus containing an anti-MCAM shRNA (MCAM-KD) or an empty vector (Control) and two days after reaching confluence were induced to differentiate. C/EBPβ (**A**), β-catenin (**B**) and ERK (**C**) were analyzed by Western-Blot at 24 h, 4 days and 30 min after induction, respectively, as indicated in Materials and Methods. GAPDH was used as loading control. The molecular weight corresponding to each band is indicated. (**D**) At different time points after induction *Mcam* and *PPARγ2* mRNAs were analyzed by RT-qPCR. Expression levels of each mRNA were normalized to *Rplp0* mRNA. RT-qPCR data are expressed as mean ± SD relative to Control cells at day 0 of duplicates from a representative experiment repeated three times with similar results * $p<0.05$ vs. Control cells at the same day (Student's t-test).

Table 1.

List of primers used in RT-qPCR

Primer	Sequence
mMcam-Fwd	ATTGGTGGCAAATCCTTCTG
mMcam-Rev	CACAAGGACCAATGTGAACG
mPlin1-Fwd	TGAAGGGTGTTACGGATAACG
mPlin1-Rev	TGAAGGGTTATCGATGTCTCG
mPPARγ2-Fwd	CCAGAGCATGGTGCCTTCGCT
mPPARγ2-Rev	CAGCAACCATTGGGTCAGCTC
mRplp0-Fwd	GAGGAATCAGATGAGGATATGGGA
mRplp0-Rev	AAGCAGGCTGACTTGGTTGC
mAdipoq-Fwd	CAGTGGATCTGACGACACCA
mAdipoq-Rev	CGAATGGGTACATTGGGAAC