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Research Article

Platelet lysate suppresses the expression of lipocalin-type prostaglandin D2 synthase that positively controls adipogenic differentiation of human mesenchymal stromal cells

Claudia Lange^{a,1}, Bärbel Brunswig-Spickenheier^{a,1}, Leah Eissing^{b,2}, Ludger Scheja^{b,*}

^aUniversity Medical Center Hamburg-Eppendorf, Clinic for Stem Cell Transplantation and Research Dept. Cell and Gene Therapy, Martinistr. 52, 20246 Hamburg, Germany

^bUniversity Medical Center Hamburg-Eppendorf, Dept. Biochemistry and Molecular Biology, Martinistr. 52, 20246 Hamburg, Germany

ARTICLE INFORMATION

Article Chronology:

Received 17 November 2011

Received in revised form

31 May 2012

Accepted 4 June 2012

Available online 12 June 2012

Keywords:

Bone marrow

Mesenchymal stromal cells

Adipocyte differentiation

Lipocalin-type prostaglandin D2 synthase

Platelet lysate

ABSTRACT

Mesenchymal stromal cells (MSCs) have been shown to display a considerable therapeutic potential in cellular therapies. However, harmful adipogenic maldifferentiation of transplanted MSCs may seriously threaten the success of this therapeutic approach. We have previously demonstrated that using platelet lysate (PL) instead of widely used fetal calf serum (FCS) diminished lipid accumulation in adipogenically stimulated human MSCs and identified, among others, lipocalin-type prostaglandin D2 synthase (L-PGDS) as a gene suppressed in PL-supplemented MSCs. Here, we investigated the role of PL and putatively pro-adipogenic L-PGDS in human MSC adipogenesis.

Next to strongly reduced levels of L-PGDS we show that PL-supplemented MSCs display markedly decreased expression of adipogenic master regulators and differentiation markers, both before and after induction of adipocyte differentiation. The low adipogenic differentiation capability of PL-supplemented MSCs could be partially restored by exogenous addition of L-PGDS protein. Conversely, siRNA-mediated downregulation of L-PGDS in FCS-supplemented MSCs profoundly reduced adipocyte differentiation. In contrast, inhibiting endogenous prostaglandin synthesis by aspirin did not reduce differentiation, suggesting that a mechanism such as lipid shuttling but not the prostaglandin D2 synthase activity of L-PGDS is critical for adipogenesis.

Our data demonstrate that L-PGDS is a novel pro-adipogenic factor in human MSCs which might be of relevance in adipocyte metabolism and disease. L-PGDS gene expression is a potential quality marker for human MSCs, as it might predict unwanted adipogenic differentiation after MSC transplantation.

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Abbreviations: L-PGDS, Lipocalin-type prostaglandin D2 synthase; MSCs, Mesenchymal stromal cells; PL, Platelet lysate; FCS, Fetal calf serum; PPAR γ , Peroxisome proliferator-activated receptor gamma; CEBP α , CCAAT/enhancer binding protein alpha; FABP4, Fatty acid binding protein 4; PLIN, Perilipin; GLUT4, Glucose transporter member 4; APOE, Apolipoprotein E; SREBP1, Sterol regulatory element-binding transcription factor 1; LXR α , Liver X nuclear receptor alpha; COX, Cyclooxygenase; TAF1, TATA box binding protein-associated factor 1; ABCA1, ATP-binding cassette transporter ABCA1; ABCG1, ATP-binding cassette sub-family G member 1; GAPDH, Glycerinaldehyde-3-phosphat-dehydrogenase

*Corresponding author. Fax +49 40 7410 54592.

E-mail addresses: cllange@uke.de (C. Lange), b.brunswig-spickenheier@uke.de (B. Brunswig-Spickenheier), leissing@uke.de (L. Eissing), l.scheja@uke.de (L. Scheja).

¹ Fax: +49 40 7410 53034.

² Fax +49 40 7410 54592.

Introduction

Mesenchymal stromal cells (MSCs) are multipotential non-hematopoietic progenitor cells capable of differentiating into multiple lineages of the mesenchyme. In bone marrow, these niche stromal cells provide a sheltering microenvironment controlling the proliferation and differentiation of hematopoietic stem cells [1]. Well documented immunomodulatory properties of MSCs include the inhibition of T cell proliferation as well as modulation of other immune cells, e.g., B, natural killer, dendritic, and cytotoxic T cells, maintaining them in an immature and/or arrested state [1,2]. Consistent with the promising immunological properties *in vitro*, MSCs have been shown to display a considerable therapeutic potential in pre-clinical [3–7] and clinical [8–14] studies for the treatment of neuronal degeneration, osteogenesis imperfecta, graft versus host disease, support of hematopoietic engraftment, metabolic diseases, bone and cartilage diseases as well as renal and myocardial infarction.

So far, no serious side effects or adverse effects have been reported in any of the published clinical trials. In contrast to studies in man, long-term partial maldifferentiation of intraglomerular MSCs into adipocytes, accompanied by glomerular sclerosis, have been reported in a progressive rat model of glomerulonephritis [15]. The direct injection of large numbers of MSCs into the renal artery as used in this model can result in abrupt deterioration of renal blood flow, resulting in infarcts and loss of kidney function. Because those reported maldifferentiations might threaten the further use of MSCs in cellular therapies, efforts concentrate on preclinical studies which could assure an as high as possible safety in using *in vitro* expanded MSCs.

We have previously shown that human MSCs expanded in platelet lysate (PL)-supplemented medium exhibited decreased lipid accumulation under adipogenic induction as compared to cells cultured in the presence of fetal calf serum (FCS) [16]. Still, these MSCs met all current criteria defining a MSC population [17]. Thus, on the one hand the use of PL provided a preferred expansion method by excluding xenogenic proteins and transfer of potentially dangerous contaminations. On the other hand, the reduced adipogenic potential provided by this method might contribute to an increased safety in using MSCs for cellular regeneration in several applications.

In our previous experiments we performed microarray analyses and identified genes differentially regulated by FCS versus PL culture supplementation. Interestingly, we observed that lipocalin-type prostaglandin D2 synthase (L-PGDS), a putative regulator of adipogenesis, was several fold less expressed in MSCs expanded with PL compared to FCS [16]. The goals of the present study were to confirm the robustness of the anti-adipogenic effect of PL using MSCs from additional donors, and to gain mechanistic insight how PL modulates adipogenesis by studying adipogenic and lipogenic genes. In addition, we tested the functional role of L-PGDS in adipogenesis of MSCs.

Materials and methods

Materials

Plastic cell culture materials were obtained from Gibco (Karlsruhe, Germany), cell culture reagents and anti-GAPDH antibody

from Novus Biologicals (Acris, Herford, Germany), L-PGDS protein and anti-L-PGDS antibody from Cayman Chemical (Ann Arbor, MI, USA), Reverse Transcriptase, OptiMEM I and the C18 fatty acid analog BODIPY[®] 500/510C1, C12 (BODIPY) from Invitrogen (Darmstadt, Germany), RNeasy Mini Kit and Quantitect predesigned L-PGDS primers from Qiagen (Hilden, Germany), TaqMan Assay-on-Demand primer sets from Applied Biosystems (Darmstadt, Germany), Microcon-membranes from Millipore (Schwalbach, Germany), siRNA from Ambion (Austin, TX, USA), DharmaFECT from Fisher Scientific (Schwerte, Germany), SYBR Premix Ex Taq from Takara Bio (Möbitec, Göttingen, Germany), protease inhibitor mix from Roche (Roche, Mannheim, Germany), chemiluminescence reagent RPN2132 from GE Healthcare (GE Healthcare, Munich, Germany) and Protran nitrocellulose membrane from Whatman (Whatman Schleicher-Schüll, Dassel Germany). AKT and pAKT antibodies were purchased from Cell Signaling Technology (New England Biolabs GmbH, Frankfurt, Germany).

Isolation and expansion of human MSCs

Bone marrow samples (BM) from healthy donors were collected after informed consent according to the hospital's guidelines approved by the Hamburg Ethics Committee and processed as described [16]. Briefly, MSCs were expanded in parallel in growth medium containing either 10% FCS or 5% PL. Cells were fed twice a week with fresh medium and incubated until reaching confluency of $\approx 90\%$. At this time point the cultures were designated as passage 0 (P0). Cells were detached with 0.05% Trypsin-EDTA and seeded at 500 cells/cm² as P1 or frozen in liquid nitrogen until further use. Human MSCs of passage 1 were characterized for expression of surface markers CD90, CD105, CD59, CD45 and CD34 and multipotential differentiation capabilities into adipogenic, osteogenic and chondrogenic lineages as described [18].

Adipogenic differentiation and quantification of lipid staining

Parallel P2-P3 cultures of FCS- and PL-supplemented MSCs of 3 individual donors were differentiated into adipocytes as described [18]. Briefly, confluent MSCs were repeatedly treated in differentiation cycles consisting of growth medium supplemented with 1 μ M dexamethasone, 0.5 mM isobutylmethylxanthine, 100 μ M indomethacin, and 10 μ M insulin (differentiation medium) for 2–3 days, unless indicated otherwise, followed by 1 day with insulin in growth medium only (maintenance medium). In each experiment the last differentiation cycle was not completed and cells were harvested after incubation with differentiation medium. For lipid staining, cells were fixed with 4% paraformaldehyde for 1 h at room temperature (RT), washed 3 times with 60% isopropanol, air-dried and stained with 0.35% Sudan Red in isopropanol for 10 min. After 4 washes with aqua dest. the cells were air-dried again. Sudan Red was dissolved in 500 μ l pure isopropanol for 10 min and the optical density (OD) determined at 500 nm. Pure isopropanol was used as control.

Expression of adipocyte genes

Parallel P2-P3 cultures of FCS- and PL-supplemented MSCs of 5 individual donors were differentiated into adipocytes for 2 weeks applying 4 differentiation cycles. Before and after this period, total RNA was isolated and purified using RNeasy Mini Kit. Quantity and quality of RNA were determined with NanoDrop 1000 (Peqlab, Erlangen, Germany). Complementary DNA was synthesized using SuperScript[®] III Reverse Transcriptase. Quantitative real-time PCR (qPCR) reactions for indicated genes (Table S1) were performed on a 7900HT sequence detection system (Applied Biosystems, Darmstadt, Germany) using TaqMan Assay-on-Demand primer sets (Table S1). Gene expressions were calculated by the $\Delta\Delta C_t$ method as copies normalized to 10^4 copies of the house keeping gene TAF1 (hereafter abbreviated as copy number).

Time course of L-PGDS mRNA and protein expression during adipogenic differentiation

To evaluate the time course of L-PGDS gene and protein expressions within a 2 weeks course of adipogenic differentiation, both PL-MSCs and FCS-MSCs from 3 individual donors were differentiated for 0 (undifferentiated control), 3, 7 or 14 days as described above. Cells were lysed in RIPA buffer (20 mM Tris-HCl, 50 mM NaCl, 5 mM EDTA, 10 mM Sodium pyrophosphate, 50 mM NaF, 1% Nonidet P40, 1% SDS, 1 mM Na_3VO_4) containing complete Mini protease inhibitor mix. Part of lysed cells was used for RNA isolation, cDNA synthesis and subsequent qPCR for L-PGDS and FABP4 as described above.

For western blotting, equal amounts of protein (12.5 μg) were separated on 4–12% SDS-PAGE and blotted onto Protran nitrocellulose membrane. Membranes were blocked for 2 h at RT with 5% milkpowder before incubation overnight at 4 °C with anti-L-PGDS antibody (1:200). Thereafter, membranes were incubated for 1.5 h at RT with horseradish-peroxidase conjugated second antibody (1:5000), followed by detection using enhanced chemiluminescence reagent RPN2132. For loading control, membranes were incubated with anti-GAPDH (1:10,000).

Phospho-AKT insulin signaling

PL- and FCS-MSCs from 3 individual donors were incubated for 15 min with insulin-containing differentiation medium. As non-induced control, incubation was continued with growth medium only. Cells were lysed and proteins (8.5 μg) subjected to Western blotting and detection as described above. The blot was probed with a primary anti-phospho-AKT antibody (1:1000) which recognizes AKT phosphorylated at Ser473. To check for equal protein loading, replicate blots were incubated with an anti-AKT antibody (1:1000).

COX inhibition in adipogenic differentiation

FCS-MSCs from 3 individual donors were expanded for 7 day in the presence (group 3 and 4) or absence (group 1 and 2) of 500 μM Aspirin as COX inhibitor. Subsequently, adipogenic differentiation was carried out either with (group 2, 3 and 4) or without (group 1) Aspirin. In group 4, indomethacin was omitted from the differentiation medium. After 2 weeks applying 4 differentiation cycles, expressions of the adipocyte genes

PPAR γ , CEBP α , FABP4, PLIN as well as L-PGDS were examined by qPCR. In parallel, cells were labeled for 3 h with 10 μM BODIPY and staining documented with an IX-81 Olympus inverted fluorescence microscope. BODIPY is a C18 fatty acid analog which readily incorporates into cellular lipids and is used to label lipid droplets in cultured adipocytes.

Stimulation of lipid accumulation by recombinant L-PGDS protein

L-PGDS protein was purified over an Amicon-membrane to remove glycerol. PL-supplemented MSCs of P3 from 3 individual donors were adipogenically differentiated for 1 week in wells of a 24-well plate. Recombinant L-PGDS protein was then added at concentrations of 50, 25, 12.5 or 0 $\mu\text{g}/\text{ml}$ to the differentiation medium at d0, followed by feeding with maintenance medium at d4. At d5 cells were again fed with differentiation medium containing recombinant L-PGDS at the concentrations described above. As a negative control, 30 min heat (95 °C) inactivated (hi) protein at a concentration of 50 $\mu\text{g}/\text{ml}$ was used. After a total period of 10 days, adipocytes were labeled for 3 h with 10 μM BODIPY. Images were captured using an IX-81 Olympus inverted microscope. Subsequently, total RNA was isolated and the expression of adipocyte genes PPAR γ , CEBP α , FABP4, PLIN and GLUT4 was determined as described above.

siRNA mediated downregulation of L-PGDS

FCS-supplemented MSCs from 5 individual donors of passages 2 to 4 were cultured in 24-well plates until 40% confluency was reached. This time point at which cells were transfected with small interfering RNA (siRNA) was designated day 0 (d0). Two different Silencer[®] Select Pre-designed siRNAs targeting human L-PGDS, S11446 and S11447, or one scrambled siRNA not matching any of the human genes were transfected into MSCs using DharmaFECT 1. Transfection was performed according to the manufacturer's instructions. In brief, DharmaFECT agent and either siRNAs or scrambled siRNA were diluted with OptiMEM 1 and preincubated for 20 min at RT (transfection solution). Final concentration of 25 nM siRNA was tested beforehand to give optimal downregulation of the L-PGDS expression and was used throughout all experiments. Final DharmaFECT concentration did not exceed 0.2%. FCS-supplemented MSCs were incubated with the transfection solution for 24 h. This procedure was conducted three times in the course of adipogenic differentiation. As controls, cells were either sham-transfected with DharmaFECT only or kept in OptiMEM 1.

After each round of L-PGDS silencing and at the end of the experimental period cells were harvested, total RNA isolated and cDNA synthesized as described above. qPCR reactions for L-PGDS gene expression were performed on a Mx3000P (Stratagene, Amsterdam, NL) using 10 ng of cDNA, SYBR Premix Ex Taq, and 200 nM GAPDH primers as housekeeping gene (forward: 5'-ACCAGGGCTGCTTTAACTCTGG-3', reverse: 5'-CACTTGATTTTGAGGGATCTCG-3') or Quantitect predesigned L-PGDS primers. Cycling conditions were 30 cycles (95 °C, 15", 55 °C, 30"; 72 °C, 30") in case of GAPDH and 40 cycles (95 °C, 15"; 58 °C, 30"; 72 °C, 30") in case of L-PGDS determination. Amplification reaction was followed by a melting curve analysis to verify product specificity. The threshold cycle (C_t) was determined for each reaction by the MaxPro Software. Relative expression of L-PGDS was calculated by the $\Delta\Delta C_t$ method and normalized for GAPDH as housekeeping gene.

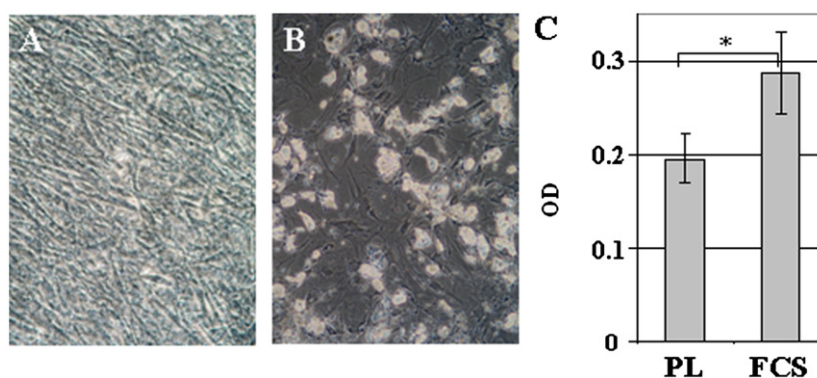


Fig. 1 – Quantification of fat accumulation in adipogenically differentiated MSCs. Differentiated PL-supplemented MSCs (A) show minimal fat accumulation, whereas in FCS-supplemented MSCs (B) large amounts of fat cells are present after 2 weeks differentiation, applying 4 differentiation cycles. One typical example out of 4 individual differentiated MSC preparations is shown. Original magnification $\times 100$. (C) The different amounts of lipid droplets were reflected in optical densities of eluted Sudan Red from stained cultures (mean $\pm$ SD of 4 experiments). * $p=0.00007$.

At the end of the siRNA/differentiation protocol, on day 11, expression of adipocyte genes was determined by TaqMan qPCR as described above. In identical parallel FCS-cultures, lipid stainings were performed and documented in photographs.

LXR activation

PL-MSCs from 3 individual donors were expanded to near confluency. LXR was activated using the LXR agonist TO901317 at 10 μ M for 0, 12 or 48 h and gene expressions of L-PGDS as well as ABCA1, ABCG1 and SREBP1 were analyzed by TaqMan qPCR as described above.

Statistics

For statistical analysis, the unpaired and two-tailed Student's *t*-test was applied, using log transformation when values were not normally distributed. *P*-values <0.05 were considered significant.

Results

FCS favors fat accumulation and adipocyte differentiation of human MSCs

Previously we have shown that PL as additive during MSC expansion gives rise to profoundly less accumulation of lipid droplets upon adipogenic induction [16]. Here, we repeated these experiments using MSCs from different donors and quantified Sudan Red stained lipids formed within 2 weeks of adipogenic differentiation (Fig. 1). Confirming our previous results, we observed significantly reduced lipid accumulation in MSCs which were expanded and differentiated in PL compared to FCS-supplemented cultures.

Several master regulators of adipogenesis and lipid synthesis are responsible for lipid accumulation in adipocytes during the course of differentiation from precursors [19–21]. In order to further characterize the role of PL in adipogenic differentiation of MSCs, we determined the expression of adipocyte genes and regulators of lipid synthesis (lipogenesis) before onset of and

after performing the adipogenic differentiation protocol in MSCs isolated from 5 different human donors. In the presence of FCS, the adipogenic protocol led to a strong increase in the expression of the adipocyte master regulators PPAR γ^3 and CEBP α , as well as the adipocyte markers FABP4, PLIN, GLUT4 and APOE, demonstrating efficient adipogenic differentiation. The lipogenic transcription factors SREBP1 and LXR α were also upregulated (Fig. 2A). In the presence of PL, the adipocyte master regulators and markers were induced as well, however to a much smaller degree compared to FCS-supplemented cultures, resulting in 5–100 fold lower expression after adipogenic differentiation.

LXR α was significantly induced in PL-supplemented cells, whereas SREBP1 was unaltered by the adipogenic protocol. Taken together, the use of PL for expanding and differentiating MSCs resulted in reduced adipocyte differentiation after adipogenic induction. Lower expression of adipocyte genes was already present in expanded cultures before adipogenic stimulation (Fig. S1), indicating that even low-level, spontaneous adipocyte differentiation was less pronounced on PL.

L-PGDS in FCS-supplemented MSCs shows higher expression but no upregulation upon adipogenic stimulation

Previously, it was reported that L-PGDS is important for differentiation of murine 3T3.L1 adipocytes [22]. Asking whether increased L-PGDS expression might be responsible for the observed higher adipocyte differentiation of FCS-supplemented MSC, we quantified the expression of L-PGDS in MSCs from 5 donors. Confirming our previous microarray results, L-PGDS expression was significantly higher in MSCs expanded in FCS versus PL (Fig. 2A). Fujimori et al. [22] reported that L-PGDS was induced during differentiation of 3T3.L1 adipocytes. L-PGDS expression was, however, not higher in FCS-supplemented MSCs after 2 weeks of differentiation (Fig. 2A), and only a small upwards trend was observed in PL-supplemented cultures, which was due to a single donor (not shown). In a separate experiment, using MSCs from 3 different donors, we examined more closely

³ See abbreviations.

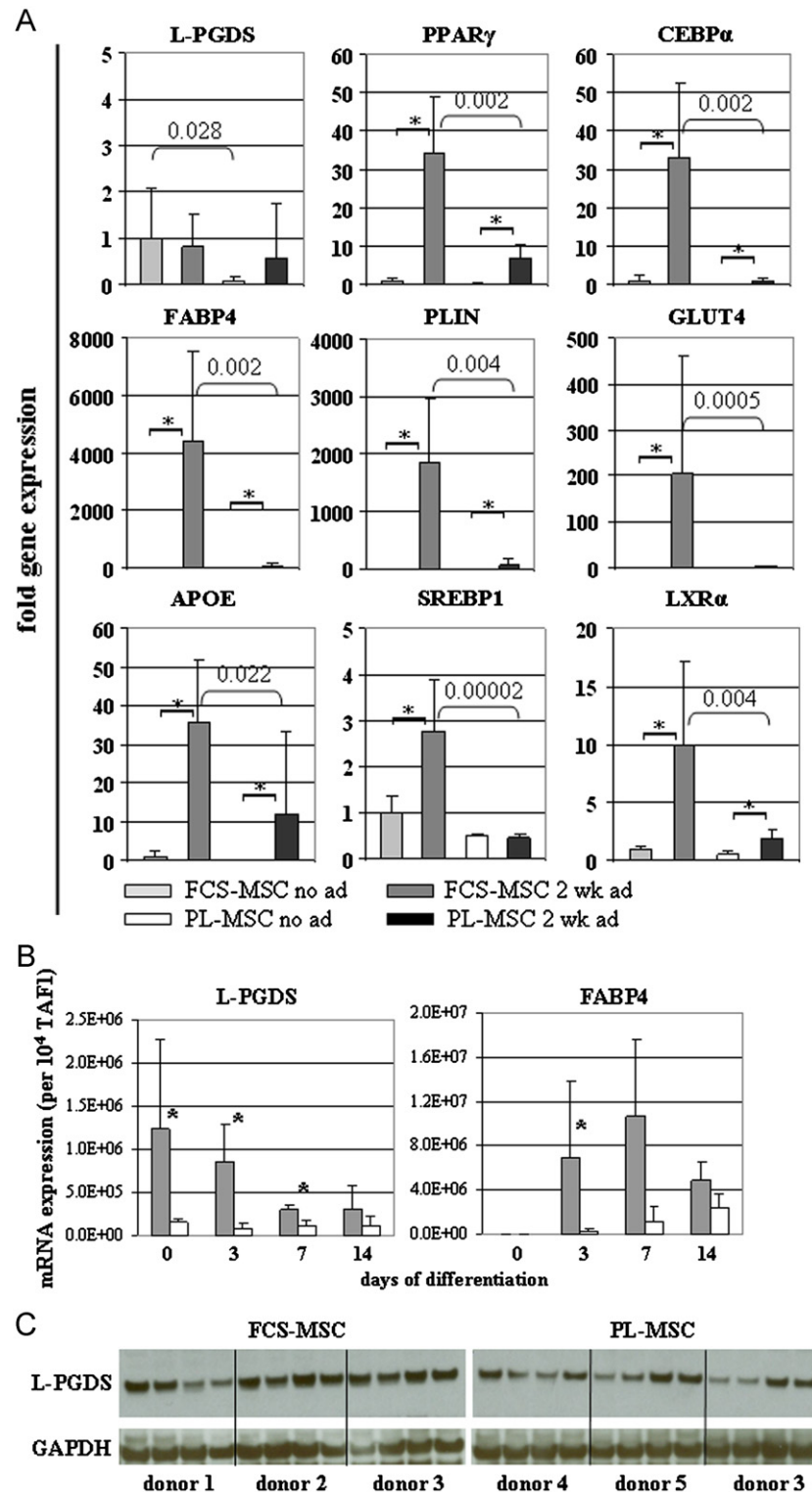


Fig. 2 – Adipogenic and lipogenic gene and protein expressions before and after induction of adipocyte differentiation. (A) FCS- or PL-supplemented MSCs of passage 3 were harvested for quantitative PCR before (no ad) or after adipogenic differentiation for 2 weeks (2 wk ad) applying 4 differentiation cycles as described in Methods. Average gene expression of non-induced FCS-supplemented MSCs was defined as 1 and all other values were calculated relative to this mean (fold). Shown are mean $\pm$ SD of 5 individual donors. T-test was done using copy numbers, **p*-values above brackets ≤ 0.005 , except for CEBP α in PL-supplemented MSCs, no ad versus 2 wk ad: *p*=0.024. (B) Time course of mRNA expression during an adipocytic induction of 2 weeks. Shown are mean $\pm$ SD of 3 individual MSC preparations differentiated in FCS (gray) or PL (white) containing medium. T-test was done using copy numbers, **p*<0.05 for FCS-MSCs versus PL-MSCs. (C) Time course of L-PGDS protein expression during an adipocytic induction of 2 weeks (from left to right: d0, d3, d7 and d14) using 3 individual donors. Anti-GAPDH was used to demonstrate equal loading.

the time course of L-PGDS expression during the two week period. We found a constant low L-PGDS mRNA level in PL-MSCs (Fig. 2B). FCS-MSCs had significantly higher L-PGDS mRNA expression for almost the whole period (Fig. 2B). L-PGDS protein levels, determined by Westernblot analysis, were also higher in FCS-MSCs than in PL-MSCs (Fig. 2C). L-PGDS mRNA in FCS-hMSC showed some downregulation over the differentiation period (Fig. 2B), which was however not present at the protein level (Fig. 2C). FABP4 mRNA was strongly induced by the differentiation protocol, demonstrating proper adipocyte differentiation in this experiment (Fig. 2B).

Recombinant L-PGDS partially restores adipocyte formation in PL-MSCs

The low expression of L-PGDS in PL-supplemented cultures suggested that low L-PGDS activity was responsible for the reduced adipogenesis in PL-supplemented cultures. In order to

test this hypothesis, we directly investigated the influence of exogenously added recombinant L-PGDS protein on adipogenesis in PL-supplemented MSCs. Recombinant L-PGDS has previously been shown to exert significant effects on cells [23] and this approach was chosen to prevent potential problems arising from ectopic gene expression in the primary MSCs. The addition of the L-PGDS protein into PL-supplemented differentiation cultures for 10 day caused a visible lipid accumulation in PL-supplemented MSCs (Fig. 3). This effect was dose-dependent and maximal at a concentration of 50 $\mu\text{g/ml}$. Heat inactivated L-PGDS protein (50 hi), applied at the maximum concentration, showed little lipid accumulation comparable to the untreated control. In order to learn if the observed increased lipid accumulation was due to more adipocyte differentiation, the expression of adipocyte markers was measured. The adipocyte genes PLIN and GLUT4 were dose-dependently increased by addition of recombinant L-PGDS. (Fig. 3), whereas the other adipocyte genes tested (PPAR γ , CEBP α and FABP4) were unchanged (not shown). Thus,

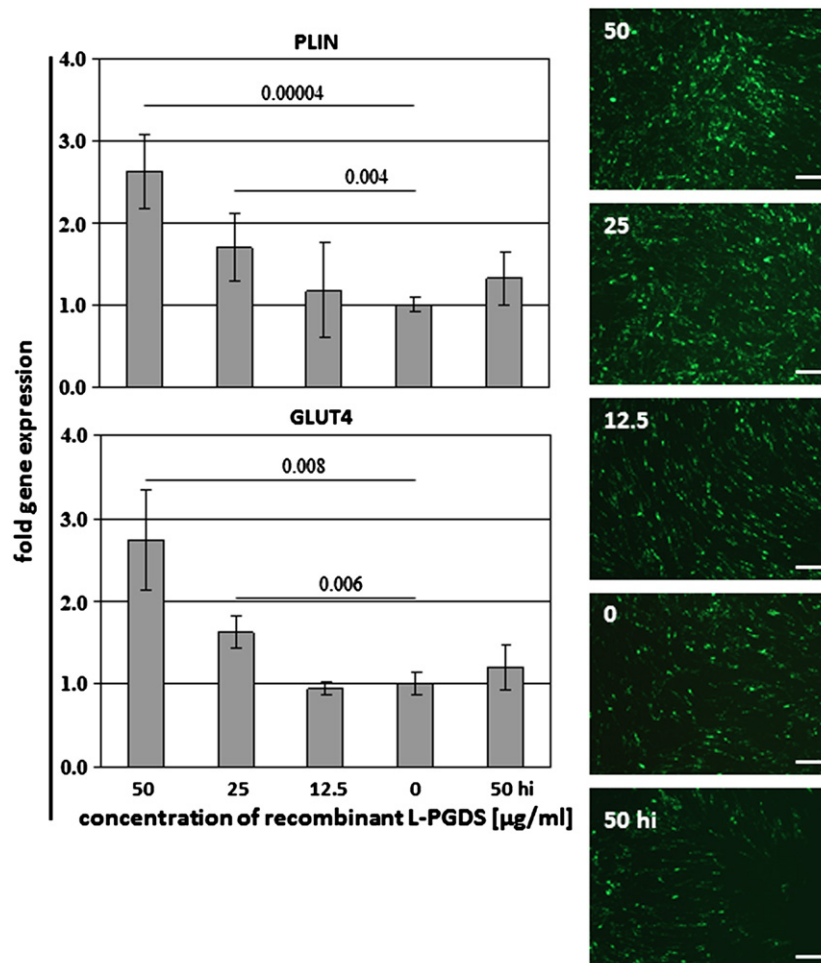


Fig. 3 – L-PGDS protein partially restores adipocyte formation. Confluent PL-supplemented MSCs of 3 individual donors were incubated with exogenously added L-PGDS protein in differentiation medium without (0) or with indicated increasing amounts of L-PGDS protein or 50 $\mu\text{g/ml}$ heat inactivated protein (50 hi) for 10 day applying 2 differentiation cycles. Afterwards, all wells were stained with BODIPY as described in Methods. Shown is one example out of three of BODIPY stained PL-MSC (right panel). Subsequently, RNA was isolated and gene expressions of PPAR γ , CEBP α , FABP4 (no significant changes, not shown), PLIN and GLUT4 tested with qPCR (left panel). Gene expressions are depicted as fold increase in comparison to gene expressions in the absence of added L-PGDS (0 $\mu\text{g/ml}$). T-test was done using copy numbers, *p*-values above bars. Data are shown as mean $\pm$ SD of 3 individual donors. Original magnification $\times 40$; bar = 200 nm.

recombinant L-PGDS can partially restore the adipogenic differentiation in PL-supplemented MSCs.

L-PGDS silencing suppresses adipogenic differentiation in the presence of FCS

Next we investigated whether the hypothesized role of L-PGDS in adipogenesis can be demonstrated in FCS-supplemented MSCs by loss-of-function experiments. To this end, we downregulated L-PGDS by repeatedly applying siRNA following the protocol shown in Fig. 4. We specifically chose a repeated downregulation to avoid potential insertional mutagenesis and change of cell properties connected with stable shRNA transfection. We found no detrimental effect of repeated transfections either on cell morphology or on cell growth, and demonstrated that the expression of L-PGDS was reliably inhibited by >80% compared to the scrambled siRNA group (Fig. 5A). The siRNA protocol did not interfere in a major way with the adipocyte differentiation protocol, as demonstrated by no significantly decreased adipocyte marker expression after three rounds of scrambled siRNA application compared with untreated controls (Fig. 5B).

In order to study the effect of L-PGDS downregulation on adipocyte differentiation, we determined lipid accumulation in L-PGDS-silenced FCS-MSCs and controls. Sudan Red staining revealed an apparent loss of differentiation capability after L-PGDS silencing, evidenced by only single small fat droplets similar to PL-supplemented MSC differentiation cultures. All control cultures, treated with scrambled RNA, DharmaFECT alone or left untreated, demonstrated comparable adipocyte formation, with a degree typical for FCS-supplemented cultures (Fig. 5C).

In line with our hypothesis that L-PGDS is essential for efficient adipocyte differentiation in the MSC cultures we found downregulation of several adipocyte genes in L-PGDS knockdown compared to scrambled control cultures (Fig. 5D). The adipogenic master transcription factors PPAR γ and CEBP α and the adipocyte differentiation markers FABP4, GLUT4 and PLIN were downregulated by at least 50% after 3 rounds of L-PGDS knockdown. The regulator of lipid synthesis SREBP1 was not significantly reduced (not shown).

No defect in growth factor or insulin signaling in PL-MSCs

Having confirmed the weaker differentiation of PL-MSCs, we looked into potential mechanisms underlying this effect. Growth factors such as PDGF, EGF, IGF-I and insulin are present in both PL and FCS. Also, they have been demonstrated to be critical for adipocyte differentiation [24]. Asking whether decreased growth factor action was responsible for the lower differentiation of PL-MSCs, we measured serine 473 phosphorylation of the tyrosine kinase downstream target protein kinase B (AKT) as a surrogate marker for signaling. In two of the 3 donors tested, a higher basal AKT phosphorylation level was found in PL-MSCs compared to FCS-MSCs (Fig. 6), most likely reflecting the increased proliferation rate of PL-MSCs compared to FCS-MSCs (data not shown). Furthermore, addition of differentiation medium, which includes a high concentration of insulin, increased AKT phosphorylation to a similar degree in PL-MSCs as it did in FCS-MSCs (Fig. 6). Thus, no defect in basal or insulin-stimulated growth factor signaling was apparent in PL-MSCs and it is unlikely that defective growth factor signaling is a reason for reduced differentiation of PL-MSCs.

Liver X receptor can induce L-PGDS in MSC but is unlikely to mediate the FCS effect on L-PGDS

Ligands of the liver X receptor (LXR) have been shown to enhance L-PGDS expression in mouse preadipocytes [22]. In order to test whether L-PGDS is an LXR target gene also in human cells and whether LXR is potentially involved in the higher L-PGDS expression in FCS-MSC, we examined the effect of the pharmacological LXR agonist T0901317 on expression of L-PGDS in PL-MSCs. T0901317 caused a pronounced increase of the established LXR targets SREBP1, ABCA1 and ABCG1, used as positive controls, already after 12 h (Fig. 7A). L-PGDS was also induced, however, only in a delayed fashion after 48 h. Thus, it is possible that in humans LXR agonists induce L-PGDS indirectly through induction of the transcription factor SREBP1 which directly stimulates the murine L-PGDS promoter [22].

In order to assess whether increased LXR activity is part of the mechanism underlying higher expression of L-PGDS in FCS-MSC,

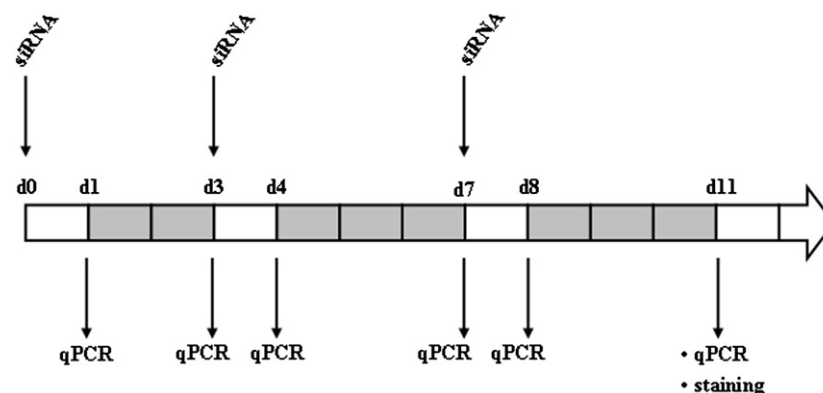


Fig. 4 – Schematic diagram of L-PGDS knockdown experiment. FCS-supplemented MSC cultures were subjected to adipogenic differentiation as described in Methods. The knockdown with siL-PGDS (siRNA) was repeatedly carried out for 24 h intervals in culture medium (d0–d1) or maintenance medium (d3–d4, d7–d8), alternating with differentiation medium intervals (gray boxes). Knockdown efficiency was determined by gene expression analysis (qPCR) 1 day after siRNA application and immediately before the next knockdown. Within 11 day, 3 knockdown rounds were applied and finished with gene expression analysis (qPCR) or Sudan red staining of fat accumulations (staining).

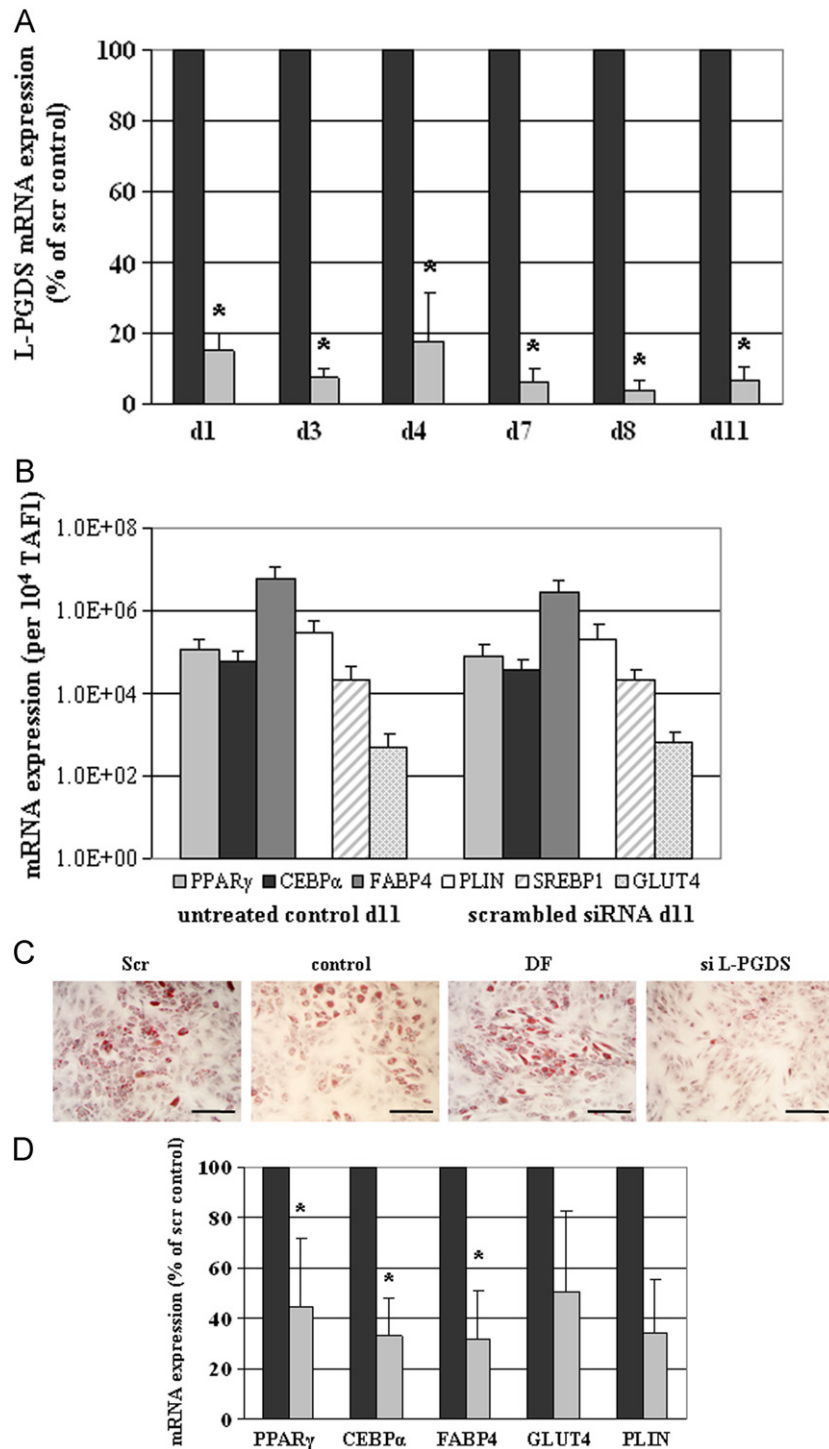


Fig. 5 – Continuous L-PGDS knockdown reduces lipid accumulation and adipocyte gene expression in FCS-MSC. (A) L-PGDS knockdown efficiency compared to treatment with scrambled RNA (black columns) which was set as 100% (mean $\pm$ SD of 5 individual donors/experiments), $*p < 10^{-6}$. **(B)** Repeated siRNA treatment does not interfere with adipocyte differentiation, as shown by gene expressions of PPAR γ , CEBP α , FABP4 and PLIN after three rounds of knockdown (scrambled siRNA d11) compared to non-transfected cells (control d11). Data represent mean of 4 independent experiments $\pm$ SD. **(C)** L-PGDS knockdown in FCS-supplemented MSCs for 11 days resulted in substantially reduced capability of lipid accumulation after adipogenic induction. All control cultures (Scr, scrambled RNA; control, induced cultures without further supplements; DF, DharmaFECT) demonstrated normal lipid accumulation. Shown is one typical example out of 5 individual experiments. Original magnification $\times 100$; bar = 200 nm. **(D)** Downregulation of adipocyte genes following L-PGDS knockdown for 11 days as compared to scrambled RNA treated cultures (black columns) which were set as 100% (mean $\pm$ SD of 5 individual donors). T-test was done using copy numbers, $*p < 0.05$.

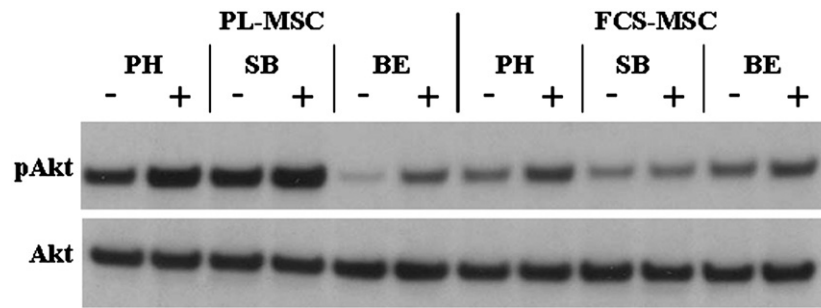


Fig. 6 – AKT phosphorylation after acute treatment with differentiation medium. MSCs from 3 individual donors (PH, SB and BE) were treated acutely with differentiation medium (labeled with +; – are untreated controls), preparing lysates after 15 min incubation. Western blotting was performed using anti-phospho AKT-Ser473 antibody. Anti-total AKT blot was used to demonstrate equal loading.

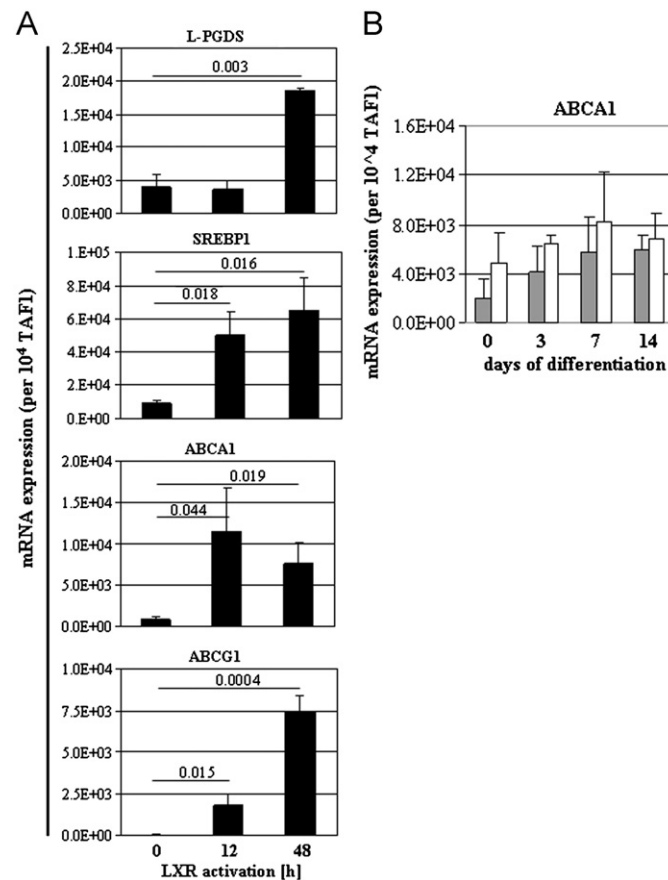


Fig. 7 – LXR α activation causes increased L-PGDS gene expression. (A) PL-expanded MSCs from 3 individual donors were cultured in duplicates for 0, 12 or 24 h in the presence of the LXR agonist TO901317 and the expression of L-PGDS, SREBP1, ABCA1 and ABCG1 was detected by qPCR. (B) Expression of the LXR target gene ABCA1 in PL-MSCs (white bars) and FCS-MSCs (gray bars) during differentiation using 3 MSC preparations from donors. T-test was done using copy numbers, *p*-values above graphs. Data represent mean $\pm$ SD.

we measured established LXR target genes in FCS-expanded MSC cultures. SREBP1 showed a trend for higher basal expression in FCS versus PL-MSC (Fig. 2A). However, ABCA1 was not expressed at a higher level in FCS- compared to PL-supplemented MSCs throughout the differentiation period (Fig. 7B), and ABCG1 was not detectable in these cultures (data not shown), arguing against a dominant role of LXR in L-PGDS expression in human FCS-MSCs.

MSCs differentiate efficiently in the presence of cyclooxygenase inhibition

L-PGDS could stimulate adipogenesis by at least two different mechanisms: (i) by providing prostaglandin D2 derivatives as PPAR γ agonists or (ii) by shuttling retinoid ligands for the PPAR γ heterodimeric partner retinoid X receptor [25]. The conventional hMSC protocol for adipogenic induction used here includes

indomethacin which is a PPAR γ agonist [26,27], however, also an efficient cyclooxygenase (COX) inhibitor at the concentrations used. As COX provides the substrate for prostaglandin D2 synthesis, it is unlikely that prostaglandin D2 derivatives are formed during the differentiation protocol. In order to confirm that prostaglandin D2 synthesis is not critical for differentiation in MSCs, we performed an experiment with MSCs from 3 donors where the COX inhibitor Aspirin was added either during the differentiation protocol only (Fig. 8, left panel, group 2) or during both expansion and

differentiation of the cells (group 3). Compared to MSCs differentiated by the conventional protocol (group 1), neither intervention altered post-differentiation lipid accumulation (Fig. 8, right panel) or expression of adipocyte genes (Fig. 8, left panel), suggesting that adipocyte differentiation in FCS-MSCs is independent of prostaglandin synthesis. In contrast, omission of indomethacin from the differentiation cocktail (Fig. 8, group 4) reduced lipid accumulation and adipocyte gene expression, suggesting that PPAR γ activation is the critical activity of indomethacin.

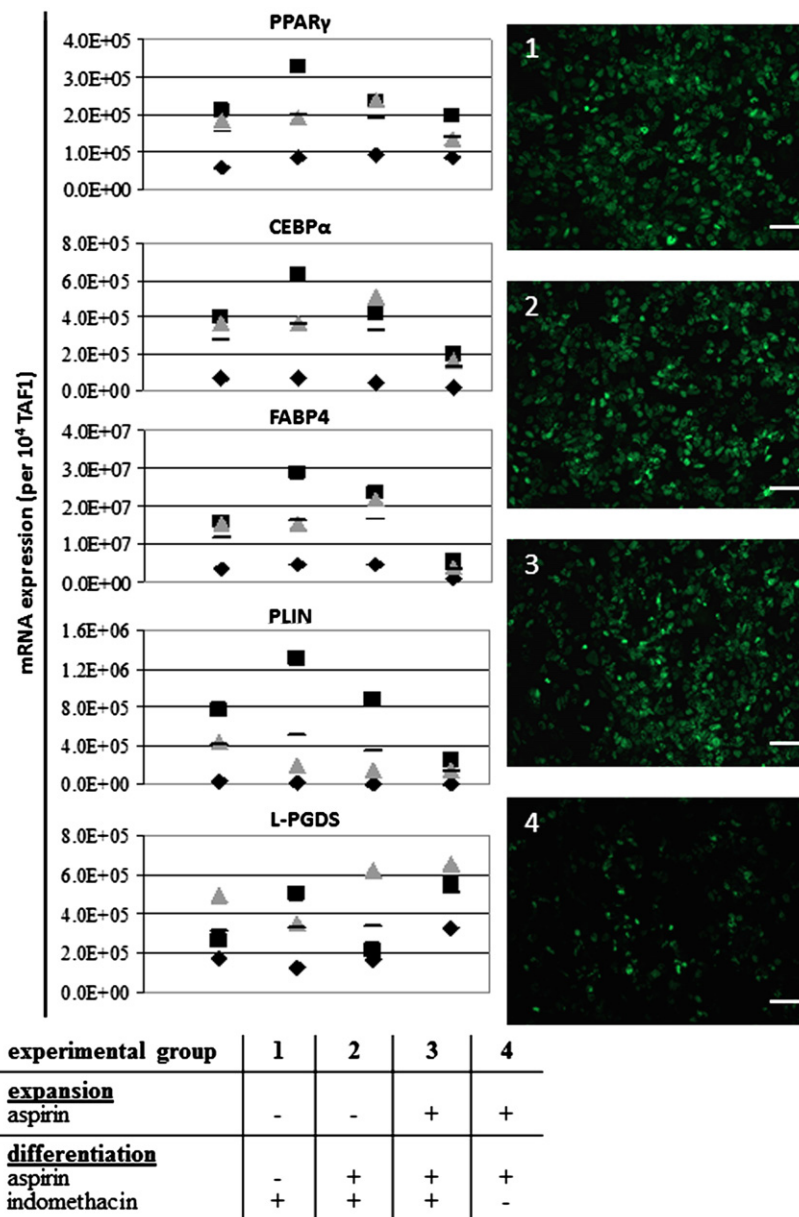


Fig. 8 – COX inhibition does not affect adipocyte differentiation of FCS-MSCs. FCS-MSCs from 3 individual donors were expanded +/- Aspirin as specific COX-inhibitor and were differentiated +/- Aspirin and +/- indomethacin for 2 weeks applying 4 differentiation cycles as indicated. Subsequently, gene expression was determined by quantitative PCR for the indicated genes as described in Methods. Gene expressions were calculated as copy numbers and are shown as individual symbols (donor 1: black squares; donor 2: gray triangle; donor 3: black rhombus). Mean of 3 individual gene expressions per experimental group are added as black bars. In parallel, adipogenically induced FCS-MSCs were stained with BODIPY. One representative example is shown (right panel). The numbers in the photos corresponds to the experimental groups shown below gene expressions. Original magnification $\times 40$; bar = 200 nm.

Discussion

Adipogenic differentiation and subsequent lipid droplet accumulation are well-organized processes involving an intricate network of transcription factors acting at different time points during differentiation [28]. In the present study we confirmed our previous finding that, in comparison to FCS, PL represents an excellent growth facilitator for human MSCs, however, compromises the differentiation capability toward adipocyte phenotype [16]. We show by gene expression analysis (Fig. 2) that the observed differences in lipid accumulation reflect adipocyte differentiation. Expression of the principal adipogenic transcription factors PPAR γ and CEBP α [21,29,30], which regulate most adipocyte genes either alone or in combination [20,21] and maintain each other's expression, was very low in PL-supplemented compared to FCS-supplemented MSCs. Consequently, the expression of genes responsible for the mature adipocyte phenotype, exemplified here by FABP4 [31,32], PLIN [33], SLC2A4 [34] and APOE [35], was much lower in PL versus FCS differentiated cultures (Fig. 2). Concurrently with less expression of the adipogenic master regulators PPAR γ and CEBP α in PL- versus FCS-supplemented MSCs, we observed lower basal expression and a lack of upregulation of SREBP1, a key transcription factor for genes involved in fatty acid, triglyceride, and phospholipid synthesis [36] which typically is upregulated during adipocyte differentiation [22]. Similarly, LXR α , another important lipogenic transcription factor [37,38] enriched in adipocytes [22] was not as much upregulated in PL-supplemented MSCs as it was in FCS-supplemented MSCs. However, the effect of PL on expression of both lipogenic transcription factors was much less pronounced than those observed for PPAR γ and CEBP α , indicating that the diminishing effect of PL on adipogenesis was mainly caused by a deficient expression of PPAR γ and CEBP α .

In the murine 3T3-L1 model, it had been previously shown that pharmacological induction of L-PGDS in 3T3-L1 cells increases adipocyte differentiation whereas siRNA-mediated L-PGDS knockdown prevented lipid accumulation, suggesting that L-PGDS is important for efficient adipocyte differentiation [22]. In contrast, work from another laboratory very recently showed that reduction of L-PGDS by stable expression of L-PGDS in antisense orientation positively regulated adipogenesis in 3T3-L1 adipocytes [39] whereas the stable expression of L-PGDS in sense orientation impaired adipogenesis [40]. The discrepancies may be due to the fact that Chowdhury et al. [39] and Hossain et al. [40] used stably transfected clones. Pre-adipocyte cell lines show clonal variability regarding adipogenic potential and one may speculate that altering L-PGDS expression level introduces a bias during clonal expansion. The role of L-PGDS in adipose tissue has also been studied in vivo by the use of knock-out mice. Although these two studies focussed on metabolic disease and not explicitly on adipocyte differentiation, they clearly demonstrate adipocyte hypertrophy in L-PGDS knockout mice [41,42]. A reduction in adipogenesis may be the underlying cause for adipocyte hypertrophy in these mice, as suggested by the observation that in vivo stimulation of adipogenesis through synthetic PPAR γ ligands reverts adipocyte hypertrophy [44]. Taken together, previous work using murine models did not provide a consistent picture regarding the role of L-PGDS in adipocyte

differentiation and more needs to be learned about the precise mechanistic role of L-PGDS in adipogenesis.

In the present study, we tested the hypothesis that reduced L-PGDS expression was causally involved in the reduced adipogenic differentiation of human primary PL-supplemented MSCs. Supporting this hypothesis, we found that L-PGDS is expressed at much lower level in expanded PL-supplemented MSC compared to FCS-supplemented MSC cultures (Fig. 2). Furthermore, the impaired adipogenesis in PL-supplemented MSCs could be partially restored within 10 day by addition of recombinant L-PGDS protein (Fig. 3). Conversely, siRNA-mediated knockdown of L-PGDS gene expression in FCS-supplemented MSCs (Fig. 5) resulted in impaired lipid accumulation. Along with L-PGDS, adipogenic master regulators and differentiation markers were also significantly downregulated, demonstrating that not only lipid accumulation but also adipogenic differentiation of MSCs was reduced efficiently. For the first time these data provide evidence that L-PGDS plays a significant role in human adipocyte differentiation.

One mechanism by which L-PGDS could stimulate adipogenesis is synthesizing prostaglandin D2-derived ligands for the adipocyte master regulator PPAR γ , as proposed for murine adipocytes [43,45]. In our experiments, prostaglandin D2 synthesis is, however, unlikely to mediate the pro-adipogenic function of L-PGDS, because the adipocyte differentiation protocol used here includes the PPAR γ ligand indomethacin which inhibits COX and, therefore, blocks prostaglandin synthesis in general at the concentrations used [46]. Furthermore, we found that additionally inhibiting COX with aspirin before and during differentiation did not reduce adipogenesis of FCS-MSCs. In summary, it appears that prostaglandin synthesis is dispensable for adipocyte differentiation in this model and that the prostaglandin D2 synthase activity of L-PGDS does not play a decisive role.

In our experiments, adding extracellular L-PGDS protein efficiently promoted adipogenesis when applied to the indomethacin-containing differentiation medium (Fig. 3). L-PGDS is a secreted protein present at substantial concentrations in extracellular fluids [47,48]. Exogenously added, extracellular L-PGDS at the concentration we used has recently been demonstrated to have significant effects on cultured cells, for example, on myocytes and adipocytes [49]. How exactly extracellular L-PGDS acts on cells is not known. A possible action is shuttling a 9-cis retinoic acid, the ligand for the PPAR γ binding partner, retinoic X receptor (RXR) [25]. Activation of both PPAR γ and RXR stimulates adipocyte differentiation [50]. Thus, shuttling of lipids such as 9-cis retinoic acid could be part of the pro-adipogenic function of L-PGDS in human MSCs.

One interesting technical aspect of our study is the efficient L-PGDS downregulation in FCS-supplemented MSCs by the use of siRNA. We were able to use repeated siRNA transfection and, yet, efficient adipocyte differentiation was achieved in the scrambled siRNA controls (Fig. 5). Gene downregulation with siRNA transfection has been reported to be active for periods of up to three days and analysis has to be carried out within this time. For longer silencing effects, stable shRNA expression is recommended. To our knowledge, these results demonstrate for the first time that repeated siRNA transfection is useful for testing differentiation in primary MSCs without need for stable siRNA integration, e.g., by using shRNA.

Taken together, in this study we provide evidence that expansion of primary human MSCs in PL-supplemented medium is

superior to the usage of FCS, assuring cell populations with a higher safety profile for use in regenerative approaches. Importantly, human MSCs already have entered the clinic for the treatment of acute kidney injury in patients who require on-pump cardiac surgery. Recently finished phase I studies demonstrated safety, feasibility and well tolerated administration of allogeneic MSCs expanded in vitro with the addition of PL [14] without any signs of maldifferentiation. We propose L-PGDS gene expression as a quality marker for human MSCs in clinical applications to avoid unwanted adipogenic differentiation which might counteract the therapeutic benefits of this promising cell population. In the present study we also provide strong evidence that L-PGDS is an important factor for induction of adipogenic differentiation in human MSCs. Our data support the hypothesis that factors inducing L-PGDS expression in MSCs are enriched in FCS compared to PL. As L-PGDS is a positive modulator of metabolic health [41,42], identification of these factors might provide novel approaches for the development of metabolic disease therapies.

Author disclosure statement

There are no commercial associations that might create a conflict of interest in connection with this article.

Acknowledgment

The authors wish to thank K. Schlagner and D. Wendt for expert technical assistance and Dr. S. Martini for support in establishing the siRNA protocol. We are grateful to A. Bartelt for critical reading of the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.yexcr.2012.06.004>.

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