



METHODS AND REAGENTS

Highly efficient differentiation of embryonic stem cells into adipocytes by ascorbic acid



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Abstract Adipocyte differentiation and function have become the major research targets due to the increasing interest in obesity and related metabolic conditions. Although, late stages of adipogenesis have been extensively studied, the early phases remain poorly understood. Here we present that supplementing ascorbic acid (AsA) to the adipogenic differentiation cocktail enables the robust and efficient differentiation of mouse embryonic stem cells (mESCs) to mature adipocytes. Such ESC-derived adipocytes mimic the gene-expression profile of subcutaneous isolated adipocytes *in vivo* remarkably well, much closer than 3T3-L1 derived ones. Moreover, the differentiated cells are in a monolayer, allowing a broad range of genome-wide studies in early and late stages of adipocyte differentiation to be performed.

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Introduction

Both the excess (obesity) and the lack/atrophy (lipodystrophy) of adipose tissue are associated with altered metabolism and lead to major diseases. In particular, the prevalence of obesity-linked diseases has increased worldwide during the

past 10 years (Savage et al., 2007). The chronic diseases associated with obesity include diabetes mellitus, arterial hypertension, metabolic syndrome and hyperlipidemia among others. In this context the adipose tissue as a target of research had been neglected for many years. However, this has changed and the adipose tissue has been recognized as an endocrine organ and source of signaling molecules and not merely a storage of fat. Therefore, several models have been set up for dissection of fat cell differentiation, key transcriptional regulators of the adipocyte differentiation program and identification of novel targets for pharmacological intervention (Rosen and MacDougald, 2006; Rosen et al., 2000; Choi

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and Tontonoz, 2013). A major limitation of essentially all of these models is that they are unable to provide information about the entire differentiation program. Hence, the earliest stages of the differentiation are unknown and therefore the sequence of events leading to preadipocyte commitment remains largely unexplored.

In principle, mouse embryonic stem cells (mESCs) and induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006) could allow one to gain insight into the early phases of any differentiation including adipogenesis on different genetic backgrounds (Rosen and MacDougald, 2006; Dani et al., 1997). However, heterogeneity of ESC cultures combined with the low efficiency of the differentiation remains the major limitation of such studies of adipogenesis (Dani et al., 1997; Schaedlich et al., 2010; Chen et al., 2007).

Ascorbic acid (AsA) has been used in various cell differentiation protocols previously (Shin et al., 2004; Takahashi et al., 2003; Kawada et al., 1990; Choi et al., 2008; Weiser et al., 2009). Recently, AsA has been recognized as a novel regulator of epigenetic control of genome activity (Chung et al., 2010; Esteban and Pei, 2012; Minor et al., 2013).

Here we provide evidence that AsA remarkably enhances adipocyte differentiation of mouse ESCs. Moreover, we provide a detailed protocol that allows using this highly reproducible, homogenous system for a variety of purposes including high-throughput genome-wide and pharmacological studies.

Materials and methods

Extended version of the listed methods and details of the materials are available in the Supplementary Information.

Cell culture and adipocyte differentiation

3T3-L1 cells and primary mouse embryonic fibroblasts (PMEFs) were cultured and differentiated as described by Kim et al. (2007) with minor modifications described in Supplementary Methods and Supplementary Table 1. mESCs E14 (kind gift of Dr. Istvan Szatmari), were cultured on PMEF feeder cells in 5% CO₂ at 37 °C. The ESC medium was prepared by supplementing DMEM Glutamax media with 15% FBS (Hyclone), 1000 U of Leukemia inhibitory factor (LIF), Penicillin/Streptomycin, Non-Essential Amino Acids and 2-mercaptoethanol. The detailed protocol for stem cell culture and adipocyte differentiation is described in the Supplementary Information. Briefly, cells were differentiated by the hanging drop method (2000 cells/drop) after 3 passages without feeder culture. Embryonic bodies (EBs) were collected at day 2 and plated in gelatin-coated dishes. A day after, the cells were treated with RA (1 μM) and AsA (12.5 μg/mL) for 3 days. From day 7 to day 11 the cells were treated with an adipogenic cocktail containing: Rosiglitazone (0.5 μM), Insulin (0.5 μg/mL), T3 (3 nM) and AsA (12.5 μg/mL). At day 12 of the differentiation the cells were replated using accutase and treated with the previously mentioned adipogenic cocktail. The cells were treated with a second adipogenic cocktail from day 15 to day 20: IBMX (0.5 μM), Dexamethasone (0.1 μM), Insulin (20 μg/mL), Rosiglitazone (0.5 μg/mL), Indomethacin (0.06 mM) and AsA (25 μg/mL). Finally from day 21 the medium was supplemented with Insulin (10 μg/mL), Rosiglitazone

(0.5 μg/mL), AsA (25 μg/mL) and T3 (3 nM) until the end of the differentiation (day 27). A summary of the adipogenic cocktails used is presented in Supplementary Table 1.

RT-qPCR

Total RNA was isolated with TRIZOL reagent (Invitrogen). cDNA synthesis was performed with Tetro Reverse Transcriptase (Bioline) according to the manufacturer's recommendation. Quantitative PCR was performed using real-time PCR (LightCycler 480II, Roche). Gene expression was quantified by the comparative C_p method and normalized to Ppia expression. Values are expressed as mean ± SD of the mean. GraphPad Prism version 5.02 was used for data interpretation and calculation of significance. The sequences of the primers are available in the Supplementary Table 2.

Oil Red O staining

Lipid droplet accumulation was assessed by Oil Red O staining visualized by microscopy or quantified by a spectrophotometer. Briefly, cells were washed with PBS and fixed with 10% formalin in PBS for at least 30 min. After washing in distilled water and 10 minutes incubation in 60% 2-propanol, cells were stained for 10 min in Oil Red O solution freshly diluted into distilled water at a ratio of 3:2, followed by three washes in distilled water. For quantification cells were allowed to dry and Oil Red O was eluted by 2-propanol for 10 min. Optical density was measured at 490 nm by a spectrophotometer-based method.

Nile Red O and DAPI staining

Stem cells were differentiated into adipocytes and at day 27 the cells were washed with PBS and fixed with 10% Formalin in PBS for at least 30 min. After washing in PBS the cells were stained with Nile Red (1 μg/mL) and DAPI (300 ng/mL) for 10 min.

Laser-scanning cytometry (LSC)

The method is explained in details in Doan et al. (2013). Briefly, cellular events were recorded applying an iCys (CompuCyt, Cambridge, MA) laser-scanning cytometer and automated cell recognition was done by CellProfiler image analyzing software to identify DAPI-stained nuclei and marked as primary objects from cells in the slide. Next, Nile Red and texture signal were used to quantitate lipid accumulation; therefore cells that contained lipid above a preset threshold value were considered adipocytes. The ratio of number of adipocytes over the total count of nuclei gave the differentiation ratio. This ratio was calculated in a region of at least 100 field images. This approach was used to count total lipid droplet number and size distribution, which were plotted using R package "ggplot2".

Microarray analysis

Microarray experiments were performed as described previously (Simandi et al., 2010). The cell culture derived adipocyte and subcutaneous isolated adipocyte (Rosenwald et al., 2013)

gene lists were obtained using GeneSpring v12.6. Heatmap and correlation plots were created using R package "pheatmap" (Team, 2011). For pairwise comparison of samples the Pearson correlation coefficients were calculated and these values were visualized on the correlation plot using the single linkage method for building the dendrogram. For identifying and removing batch effects from microarray data derived from different platforms, the R package "ber" was applied. All microarray data from this study have been submitted to the Gene Expression Omnibus (Series accession number: [GSE49176](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=xtmlfciyaakoeby&acc=GSE49176)).

The following link has been created to allow review of record [GSE49176:https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=xtmlfciyaakoeby&acc=GSE49176](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=xtmlfciyaakoeby&acc=GSE49176).

Western blot analysis

20 µg protein whole cell extract was separated by SDS-PAGE gel electrophoresis in 10 or 12.5% polyacrylamide gel and then transferred to Immobilon-P Transfer Membrane (Millipore Corp., Billerica, Massachusetts). Membranes were probed with anti-PPAR γ (81B8; Cell Signaling), anti-FABP4 (10004944; Cayman Chemical), anti-RXR α (sc-553; Santa Cruz), anti-OCT3/4 (sc-5279; Santa Cruz), and anti-actin (A2066, Sigma) antibodies, according to the manufacturer's recommendations.

Chromatin immunoprecipitation (ChIP) and sequencing

ChIP-seq experiments were carried out as previously described (Barish et al., 2012), with minor modifications. Cells in different stages of the adipocyte differentiation were cross-linked in two steps using first Disuccinimidyl glutarate (DSG) for 30 min then 1% methanol-free ultrapure formaldehyde for 10 min at room temperature. Glycine was added for 5 min in 125 mM final concentration. After fixation chromatin was sonicated with Diagenode Bioruptor to generate 200–1000 bp fragments. Chromatins were immunoprecipitated with antibodies against pre-immune IgG (Millipore, 12-370) and H3K27ac (Abcam, ab4729). The eluted DNA was purified (Qiagen, MinElute) and quantified with a Qubit fluorometer (Invitrogen). ChIP-seq libraries were prepared by Ovation Ultralow Library Systems (Nugen) according to the manufacturer's instructions.

ChIP-seq analysis

Primary analysis of the ChIP-seq raw reads has been carried out using the ChIP-seq analyze command line pipeline (Barta, 2011; Nagy et al., 2013). Peaks were predicted by MACS2 (Zhang et al., 2008). IGV was used for data visualization (Lee et al., 2012). ChIP-seq data has been submitted to the Sequence Read Archive Bioproject ID: PRJNA242495, Study accession: SRP040490, Experiment accession: SRX497240, Run accession: SRR1200451.

Glucose uptake colorimetric assay kit

The insulin dependent-glucose uptake experiments were carried out in terminally differentiated, AsA treated cell derived adipocytes with a colorimetric assay (MAK083, Sigma) according to the manufacturer's instructions.

Statistical analysis

The descriptive statistics values are presented in the plots and in the figure legends as the mean \pm SD or mean \pm SEM in the case of the large data sets (lipid droplet number and size quantification). The probability of type I error used was 0.05. ANOVA was used to compare the different treatment means. When the ANOVA null hypothesis was rejected, multiple comparisons were performed using Tukey's HSD test. Unpaired t-test was used to compare two groups.

Results and discussion

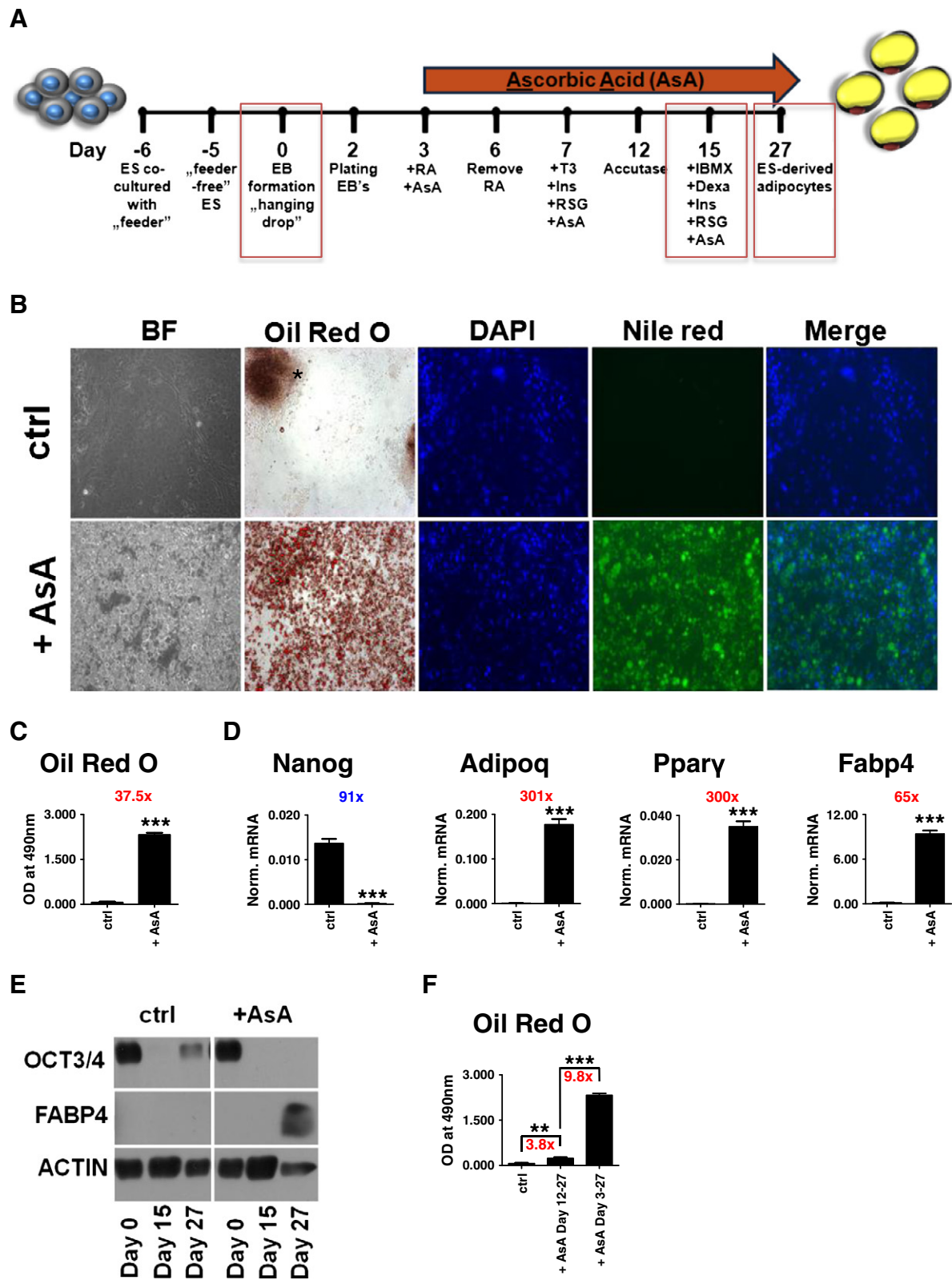
Ascorbic acid enhances stem cell to adipocyte differentiation

A previously described adipocyte differentiation protocol relies on the formation of EBs from ESCs and the usage of cocktails to differentiate the cells into adipocytes (Dani et al., 1997). After 27 days of differentiation one could typically obtain a mix of cells growing in cell clumps and containing only 15–30% adipocytes (Dani et al., 1997; Schaedlich et al., 2010; Chen et al., 2007). This variable heterogeneity hinders biochemical and/or molecular biological analyses of the differentiation process. We asked whether AsA could be used to enhance adipogenic differentiation of ESCs in this model

Figure 1 Ascorbic acid (AsA) enhances differentiation of mESC to adipocytes. A, stepwise differentiation protocol of mESCs into adipocytes. B, immunostaining of control (ctrl) and AsA treated, ESC derived adipocytes for lipid accumulation markers (Oil Red O and Nile Red) and for nuclei (DAPI) on day 27 of the adipocyte differentiation. Asterisk (*) shows an unspecific staining of an undifferentiated stem cell colony. C, quantification of Oil Red O staining as measured by spectrophotometry of the control and AsA treated cultures at the end of the differentiation (day 27). The mean, standard deviation and fold difference of four independent samples are shown. D, RT-qPCR measurement of embryonic stem cell marker (Nanog) and adipocyte markers (Adipoq, Ppar γ , Fabp4) in the terminally differentiated cultures. Experiments were carried out at least in three biological replicates. Results of a representative measurement are shown. The fold differences that are presented are calculated based on the mean and standard deviation of the qPCR technical replicates. E, protein samples were collected at the indicated time points (see also Fig. 1A red boxes). Immunoblot analysis of samples was performed using antibodies against OCT3/4 (a pluripotency marker) and FABP4 (adipocyte marker). Actin was used as a loading control. A representative experiment is shown. F, comparison of continuous (3–27) and partial (12–27) AsA treatment in the adipocyte differentiation. Results of Oil Red O quantification of samples resultant from the indicated culture conditions. ESCs were differentiated to adipocytes in the absence of AsA (ctrl) or AsA was introduced from day 3 or day 12. The mean and standard deviation of 3 independent replicates are shown. (*p = 0.05–0.01, **p = 0.01–0.001, ***p < 0.001).

described previously (Supplementary Fig. 1A). We have found that over a wide range of concentrations AsA significantly improves adipocyte differentiation (Supplementary Fig. 1B),

the highest increase was found when AsA was added from day 2 of differentiation (Supplementary Figs. 1C and D). Despite the significantly increased efficiency, the presence of cell clumps,



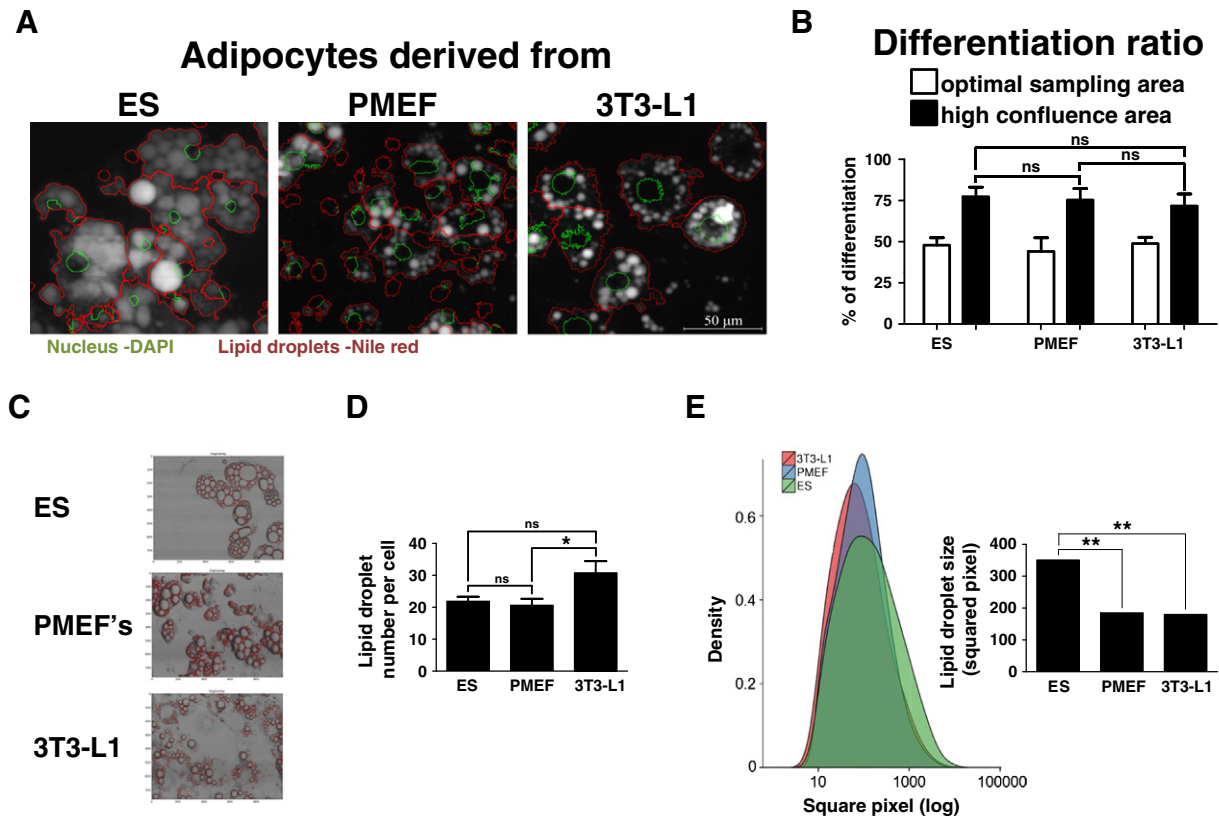


Figure 2 Adipocyte differentiation ratio measured by laser-scanning cytometry. Automatic recognition of the cellular components: lipid droplets in red line and nucleus green from ESCs, PMEF and 3T3-L1 derived adipocytes (A) in DAPI and Nile red previously stained slides. The differentiation ratio was calculated in the optimal (white bar) and high confluence (black bar) areas of scanning (B). The scanning was performed in 100 field images in at least 2 independent experiments. C, lipid droplets within the different adipocytes were recognized by Nile red staining and quantified in number and size. D, lipid droplet number per cell was quantified in ES, PMEF, and 3T3-L1 derived adipocytes and plotted. E, lipid droplet size (square pixel) distribution is plotted in a density graph (x-axis is on log10 scale). The bar graph represents the median and the SEM. Squared pixel (pixel²), 1 square pixel = 0.06125 μm^2 , pixel size is $0.25 \times 0.245 \mu\text{m}$, in 40 \times objective resolution. Inspected >200,000 lipid droplets in more than 1000 adipocyte in at least 2 independent replicates (ns $p > 0.05$, * $p = 0.05-0.01$, ** $p = 0.01-0.001$, *** $p < 0.001$).

originating from EBs, still resulted in a heterogenic, non-monolayer cell culture. To overcome this issue, we introduced accutase treatment as an additional step resulting in a monolayer differentiation. Our modified protocol, depicted in Fig. 1A, shows a better adipocyte differentiation than the previously described protocol

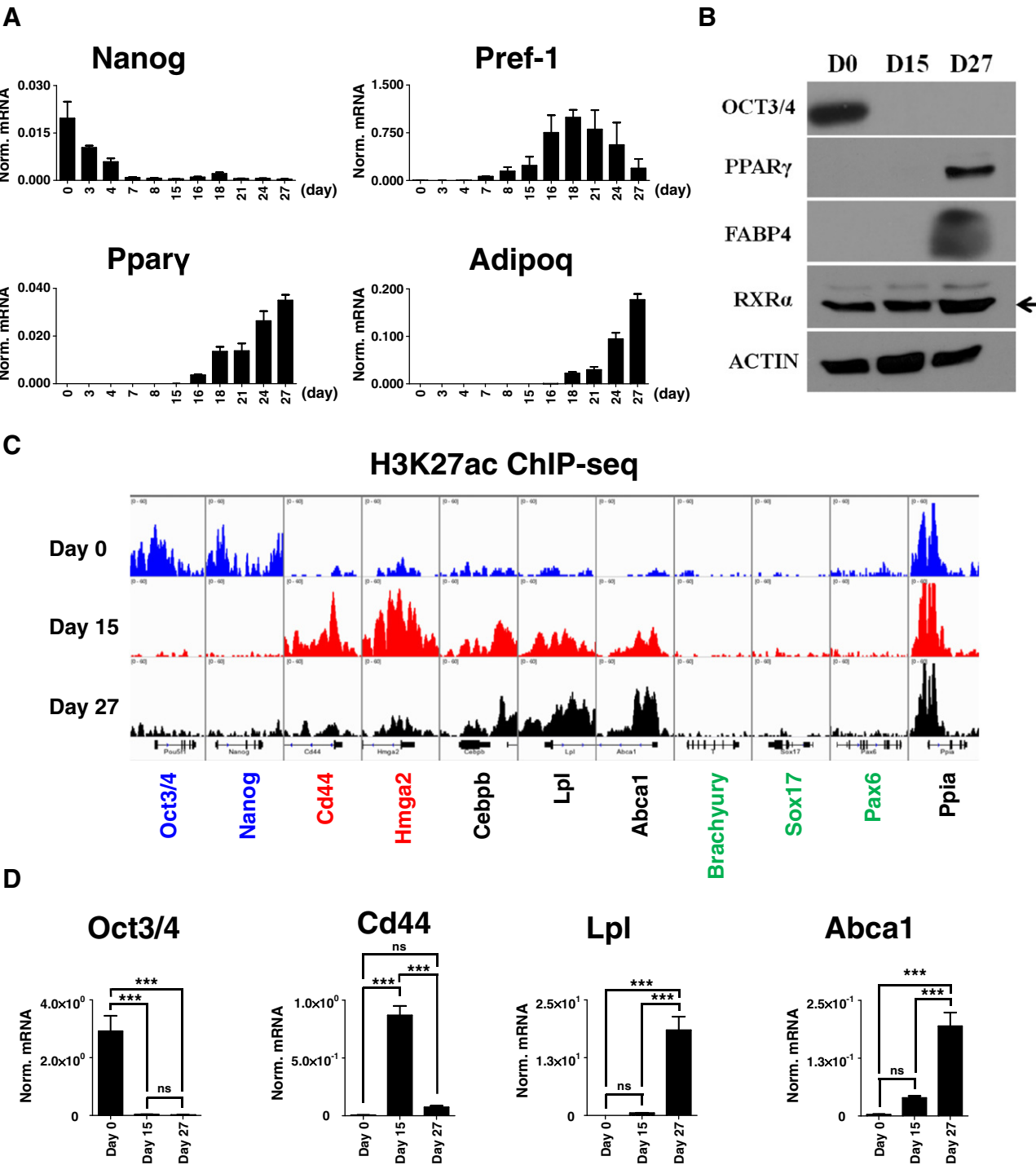
(compared in Supplementary Fig. 2). This new protocol includes: EB formation (day 0), induction of ESC differentiation by retinoic acid (RA) (day 3–day 6) and adipogenic cocktail (T3, Insulin, Rosiglitazone) (day 7–day 12), removal of the cell clumps by accutase (day 12), and treatment along with an extended adipocyte differentiation cocktail (Insulin,

Figure 3 Gene expression and epigenetic studies on ESC-derived adipocytes. A, dynamics of gene expression profile in the adipocyte differentiation. Pluripotency (Nanog), preadipocyte (Pref-1) and adipocyte (Ppar γ and Adipoq) expression was measured by RT-qPCR in the indicated time points of the differentiation. Experiments were carried out at least in 3 biological replicates. Results of a representative measurement are shown. The fold differences that are shown are calculated based on the mean and standard deviation of the RT-qPCR technical replicates. B, immunoblots of protein samples from the indicated stages of adipocyte differentiation (Fig. 1A red boxes); probed for expression of OCT3/4, PPAR γ , FABP4 and RXR α . Actin was used as a loading control. A representative experiment is shown. C, example for epigenetic reorganization of the chromatin along the adipocyte differentiation. Results of H3K27ac ChIP-seq are shown at days 0, 15 and 27. Signal intensity as visualized by IGV genome browser is shown on the genomic region of ES specific genes (Oct3/4, Nanog), mesenchymal stem cell markers (Cd44, Hmga2), and preadipocyte and adipocyte markers (C/ebp β , Lpl, Abca1). Early progenitor markers of ectoderm (Pax6), mesoderm (Brachyury) and endoderm (Sox17) are also shown. Ppia is a control region, active in each investigated cell type. D, RT-qPCR measurement of the genes Oct3/4, Cd44, Lpl, and Abca1 during adipocyte differentiation from mESC. Days 0, 15 and 27 were evaluated to correlate with the H3K27 ChIP-seq signal intensity. The mean and standard deviation of 3 independent replicates are shown (ns $p > 0.05$, * $p = 0.05-0.01$, ** $p = 0.01-0.001$, *** $p < 0.001$).

RSG, IBMX, Dexamethasone) (day 15–day 20) and from day 21 Insulin, RSG and T3. AsA was added continuously from day 2. A fully detailed protocol and a composition of differentiation cocktails used in this manuscript are available in the Supplementary Methods and Supplementary Table 1.

The addition of AsA from day 3 results in a reproducibly homogeneous adipocyte differentiation in 27 days of culture. These cells show the typical lipid accumulation markers of a mature adipocyte (Figs. 1B and C). The expression levels of

adipocyte-specific markers are at least 50-fold higher at the mRNA (Fig. 1D) and consistent with data from other approaches also at the protein level (Fig. 1E and Supplementary Fig. 3) in AsA treated cells if compared to control differentiated cells. Remarkably, without AsA treatment we can detect in the culture residual stem cell markers at day 27, suggesting that in the absence of AsA there are non-terminally differentiated dividing cells remaining in the culture and therefore reducing the homogeneity of the culture (Figs. 1B, D, E and Supplementary Fig. 3).



In addition, we tested at which differentiation stage the cells are more sensitive to the boosting effect of AsA. The results show that although AsA significantly enhances adipogenic differentiation, its presence in the late stage only partially increases the efficiency of the differentiation, suggesting that AsA mainly affects early cell fate commitment (Fig. 1F, Supplementary Fig. 1).

To determine accurately the differentiation ratio in the culture, we used the laser-scanning cytometry (LSC) technology (Rosenwald et al., 2013). This allows the analyses of monolayer cultures. In the areas where the cell density was optimal for LSC measurement the ratio of adipocytes reached at least 50% in AsA treated ESCs. As a control of measurement we also determined the differentiation of the widely used 3T3-L1 cells and PMEF after 10 and 12 days of differentiation respectively with AsA treatment and observed a similar ratio. However, the adipocytes tend to differentiate in colonies resulting in some high confluent areas. We found that in these areas the adipocyte differentiation ratio can reach up to 80% in all the cell-derived adipocytes (Figs. 2A and B). The measurements also show that the adipocytes derived from 3T3-L1 cells contain in average smaller lipid droplets, a feature of immature adipocytes (Tanaka et al., 1997; Nagayama et al., 2007). In contrast, stem cell derived adipocytes contain fewer and bigger lipid droplets (Figs. 2C, D and E).

As a functional evidence of adipocyte differentiation we found that stimulated glucose uptake and the expression level of glucose transporters (Glut1 and Glut4) are also comparable between the mESC, 3T3-L1 and PMEF derived adipocytes (Supplementary Fig. 4).

Our results demonstrate that AsA can also be used to enhance adipocyte differentiation of PMEF (Supplementary Figs. 5A–C). To determine the effective dose of AsA we tested a range of concentrations and found that the $EC_{50} = 2.81 \mu M$ ($0.49 \mu g/mL$) (Supplementary Fig. 5D). This is in agreement with the concentrations used by others in other cellular differentiation systems (Shin et al., 2004; Takahashi et al., 2003).

Adipocytes differentiated from mESC are suitable for genome-wide studies

Adipogenic differentiation appears to follow a highly ordered sequence of events (Rosen et al., 2000). However, the details of the steps of early cell fate commitment are not well understood due to the technical limitations described above. Importantly, our model system provides a novel cellular model for the field that will likely promote our understanding of the upstream molecular events during adipocyte differentiation from mESC. As far as markers of the various stages of differentiation are concerned: stem cell marker Nanog expression is gradually decreasing until it is no longer detectable at day 7, preadipocyte marker Pref-1 (also known as Dlk1) is induced but shows a decreased expression from day 18. Levels of the key adipocyte transcription factors PPAR γ and Adipoq are continuously increasing from day 16 to 18, respectively (Fig. 3A). Protein and gene expression levels of OCT3/4, PPAR γ , FABP4 show a similar pattern (Fig. 3B and Supplementary Fig. 6), suggesting that this model system is suitable for genome-wide gene expression and proteomic studies as well.

Another important feature of this improved adipocyte differentiation protocol is that it allows one to study epigenetic changes and dynamics of transcription factors along the entire differentiation process as well. In order to illustrate this we performed the analysis of the well-established histone mark of active chromatin, Histone 3K27 acetylation (H3K27ac) and compared with previously published data obtained in 3T3-L1 cells (Mikkelsen et al., 2010). H3K27ac shows a very dynamic reorganization at the chromatin level (Fig. 3C) and correlates with gene expression (Fig. 3D). The genome-wide H3K27ac data in adipocytes derived from mESC compared to the H3K27 ac from 3T3-L1 derived adipocytes showed in both cases increased acetylation levels in the close proximity of PPAR gamma binding sites and low acetylation near the insulator CTCF (Supplementary Figs. 7A and B).

mESC-derived adipocytes closely resemble *in vivo* subcutaneous isolated adipocytes

Finally, an unbiased, systematic gene expression comparison at the genome-wide level was carried out to show that the ESC derived adipocytes very closely resemble *in vivo* subcutaneous isolated adipocytes (Fig. 4A). We compared the adipocytes derived from different cell types (mESC, PMEF, 3T3-L1) to inguinal isolated adipocytes (Rosenwald et al., 2013) using gene expression data. The unbiased hierarchical cluster analysis revealed that mESC derived adipocytes are the closest to the *in vivo* isolated adipocytes. Focusing only on key markers of the various cellular stages we have found that adipocytes derived from stem cells show no remarkable differences compared to *in vivo* isolated adipocytes (Fig. 4B and Supplementary Fig. 8).

Previous studies suggested that the addition of Rosiglitazone promotes adipocyte differentiation (Kim et al., 2007; Zebisch et al., 2012; Wang et al., 2014). However, a recent study showed that the addition of Rosiglitazone might cause brown fat conversion from white fat through the stabilization of PRDM16 at protein level (Ohno et al., 2012). Thus, we carried out adipocyte differentiation experiments from mESC in the presence and absence of Rosiglitazone. In agreement with previous reports (Elabd et al., 2009; Pisani et al., 2011), differentiating mESC that is chronically exposed to Rosiglitazone expresses “browning” markers (e.g. Ucp1, Cidea) at a significant higher level than the untreated. In contrast, in the absence of PPAR gamma agonist we could detect very low levels of the brown adipocyte markers and the absence of Rosiglitazone resulted in only a slight decrease of adipocyte differentiation (Figs. 5A and B).

Conclusions

The results presented here demonstrate that the incorporation of AsA into the differentiation protocol enhances adipocyte differentiation of ESCs. Such differentiated cells can be studied by the Next Generation Sequencing (NGS) based techniques without the need of cell separation and handling. The presented model with the detailed protocol provided in the Supplementary Methods enables one to perform investigations of the transcriptional events during the entire process of cellular adipogenesis with high resolution

allowing to carry out transcriptomic as well as proteomic and lipidomic studies. Importantly, recent advances in stem cell biology allow the high-throughput genetic manipulation of mESCs and functional validation of genes identified to be important in adipocyte differentiation as well.

Adipocyte differentiation from mESC requires EB formation and ATRA dependent activation of RAR β signaling (Lee et al., 2012; Wdziekonski et al., 2007; Simandi et al., 2013) as a necessary first step for successful differentiation. Rosiglitazone treatment is dispensable from days 7 to 15, as it was shown in Fig. 5. In contrast preadipocytes can undergo adipogenesis in confluent cultures with the addition of IBMX, Insulin and Dexamethasone (Hausman et al., 2008). Although to enhance adipogenesis PPAR gamma agonist is commonly used (Kim et al., 2007; Zebisch et al., 2012; Wang et al., 2014). And in some cases as in sorted PDGFR α + preadipocytes robust adipocyte differentiation can be achieved with the only addition of Insulin (Lee et al., 2012).

The mechanism on how AsA enhances the differentiation is not well understood, however it is unlikely that the antioxidant effect of AsA is involved (Shin et al., 2004; Takahashi et al., 2003; Arrigoni and De Tullio, 2002). AsA has been postulated to contribute to other pathways relevant in adipocyte differentiation, including the regulation of the activity of HIF prolyl-hydroxylases and increasing cAMP levels and also has been linked to the

activity of the JmJc histone demethylases (Esteban and Pei, 2012; Team, 2011; Pisani et al., 2011; Arrigoni and De Tullio, 2002; Floyd et al., 2007; Farmer, 2006). This aspect of the compound's effect on cellular differentiation needs further studies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.scr.2014.04.015>.

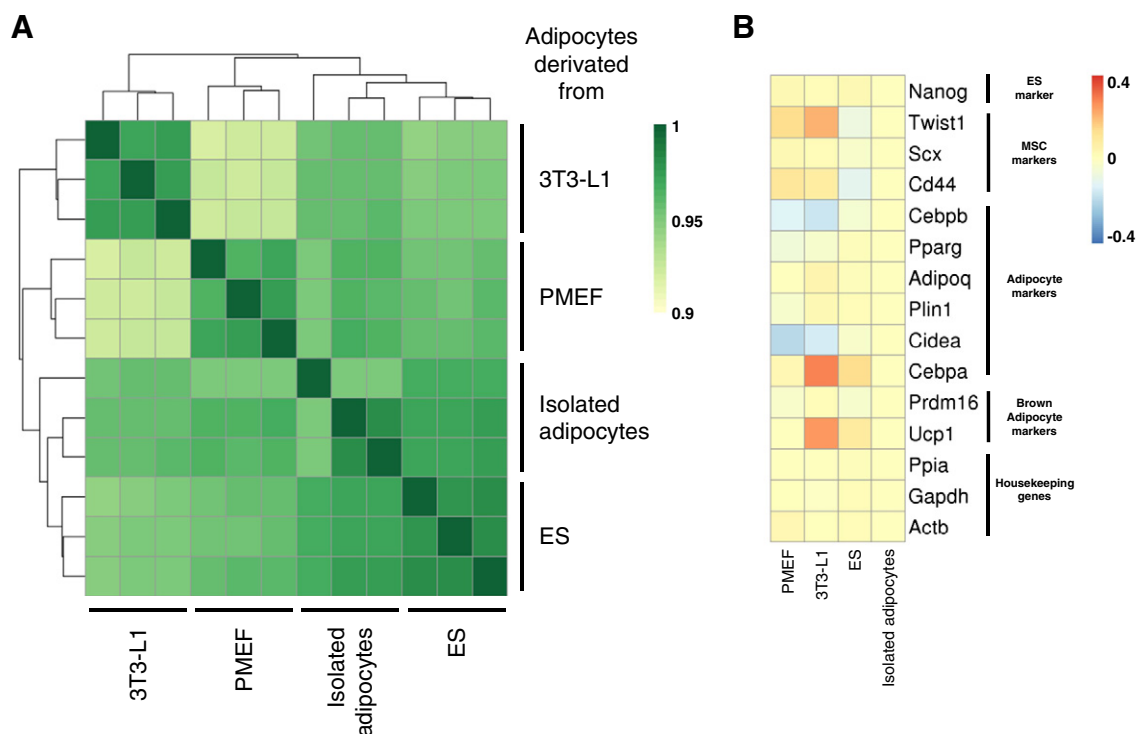


Figure 4 Genome-wide gene expression differences between different model systems of adipogenesis. A, unbiased hierarchical cluster analysis and heat map display of ESCs, PMEF, 3T3-L1-derived adipocytes *versus* subcutaneous isolated adipocytes (Rosenwald et al., 2013). 3T3-L1, PMEF and mESCs were differentiated to adipocytes under the treatment of AsA (Supplementary Table 1) and compared to the *in vivo* isolated adipocytes. Microarray experiments were carried out in biological triplicates. The overlapping genes between the different array types based on their Entrez gene ID gene list were determined. Pearson correlation was used and plotted in the correlation plot. B, comparison of embryonic stem cell (ESC), mesenchymal stem cell (MSC), adipocyte and brown adipocyte markers in the indicated cells differentiated to adipocytes. Heatmap displays stage specific markers. Red color represents upregulated genes, and blue represents downregulated genes.

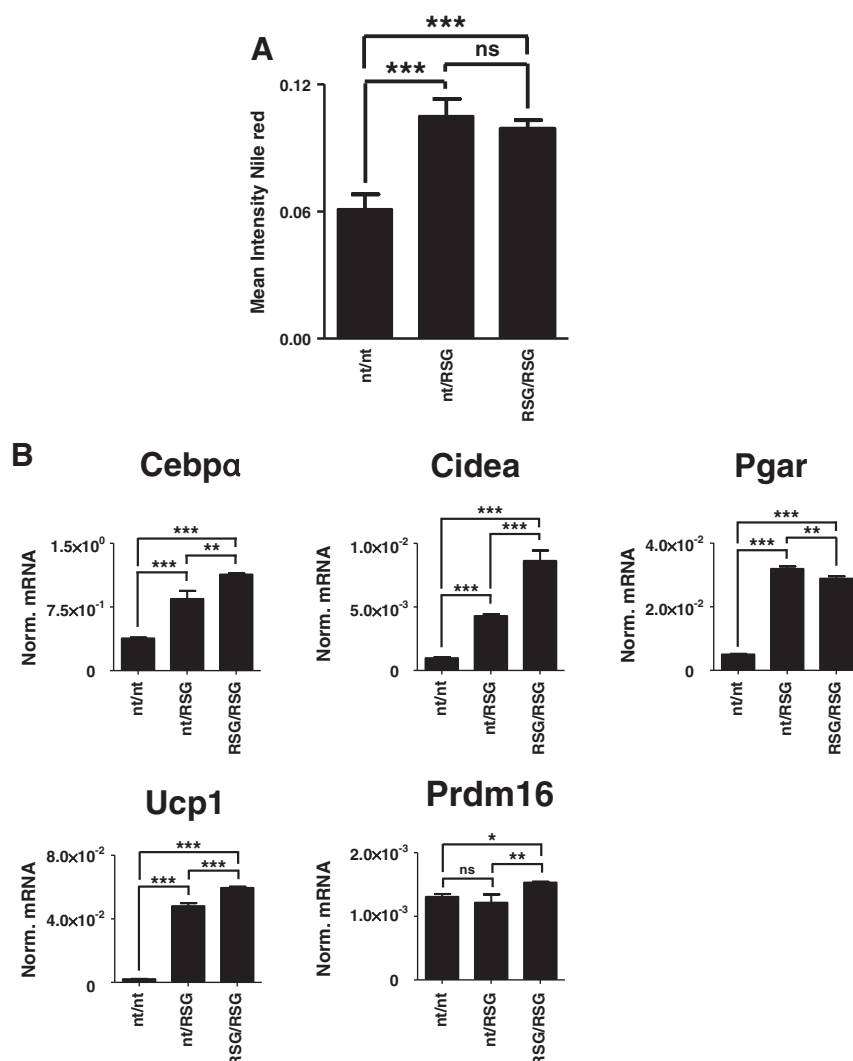


Figure 5 Rosiglitazone effect during adipocyte differentiation from mES. A, Nile red intensity measurements during laser-scanning cytometry at the end stage of adipocyte differentiation from mESC in the presence or absence of Rosiglitazone (RSG). Treatments: No RSG treatment (nt/nt), RSG treatment between day 0 to day 15 (t/nt) and continuous RSG treatment from day 0 (t/t). The mean and standard deviation of 2 independent samples are shown; scanning was performed in 100 field images. B, RT-qPCR measurement of brown adipocyte markers (Ucp1, Prdm16, Cidea), adipocyte marker (Cebpa) and PPAR gamma direct target gene (Pgar) in the terminally differentiated cultures. The mean and standard deviation of three independent replicates are shown. (* $p = 0.05$ – 0.01 , ** $p = 0.01$ – 0.001 , *** $p < 0.001$).

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