PPARγ ligand production is tightly linked to clonal expansion during initiation of adipocyte differentiation

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Abstract Adipocyte differentiation is orchestrated by the ligand-activated nuclear receptor PPARγ. Endogenous ligands comprise oxidized derivatives of arachidonic acid and structurally similar PUFA s. Although expression of PPARγ peaks in mature adipocytes, ligands are produced primarily at the onset of differentiation. Concomitant with agonist production, murine fibroblasts undergo two rounds of mitosis referred to as mitotic clonal expansion. Here we show that mouse embryonic fibroblasts deficient in either of two cell cycle inhibitors, the transcription factor p53 or its target gene encoding the cyclin-dependent kinase inhibitor p21, exhibit increased adipogenic potential. The antiadipogenic effect of p53 relied on its transcriptional activity and p21 expression but was circumvented by administration of an exogenous PPARγ agonist suggesting a linkage between cell cycling and PPARγ ligand production. Indeed, cell cycle inhibitory compounds decreased PPARγ ligand production in differentiating 3T3-L1 preadipocytes. Furthermore, these inhibitors abolished the release of arachidonic acid induced by the hormonal cocktail initiating adipogenesis. Collectively, our results suggest that murine fibroblasts require clonal expansion for PPARγ ligand production at the onset of adipocyte differentiation. — Hallenborg, P. R. K. Petersen, S. Feddersen, U. Sundekilde, J. B. Hansen, B. Blagoev, L. Madsen, and K. Kristiansen. PPARγ ligand production is tightly linked to clonal expansion during initiation of adipocyte differentiation. J. Lipid Res. 2014. 55: 2491–2500.

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Although several transcription factors are known to regulate adipocyte differentiation, PPARγ stands out as the master regulator of adipogenesis (1, 2). PPARγ belongs to the superfamily of nuclear receptors generally characterized by their two distinct transactivation domains, the ligand-dependent and the ligand-independent domains. Although it has been shown that the ligand-independent domain is able to drive adipogenesis in NIH-3T3 cells (3), a number of observations are in agreement with the notion that PPARγ ligand(s), the ligand-dependent domain, and the associated transactivation function are important for initiation of adipocyte differentiation. Thus, several PPARγ antagonists are reported to decrease adipose conversion (4–6). Furthermore, whereas ectopic expression of wild-type PPARγ restores adipocyte differentiation in PPARγ-deficient mouse embryonic fibroblasts (MEFs), a PPARγ mutant with a non-functional ligand-dependent transactivation function fails to do so (7).

PPARγ agonist production peaks during the initial stage of adipocyte differentiation where PPARγ expression is still low (6, 8). Concomitant with the generation of PPARγ agonists, differentiating murine preadipocytes undergo two rounds of cell division. This process, commonly denoted mitotic clonal expansion, has normally been regarded as a prerequisite for efficient adipose conversion (9), although it has been suggested that only entry into S-phase is necessary for initiation of differentiation (10).

The exact mechanisms linking clonal expansion to adipogenesis are unknown. It was demonstrated that entry into S-phase leads to the acquisition of functional DNA binding capability of CCAAT/enhancer binding protein (C/EBP) β through phosphorylation of certain residues (11). The delay in functional DNA binding of C/EBPβ...
compared with its boost in expression was suggested to be important for timely induction of C/EBPa and PPARγ expression. However, mutational ablation of the phosphorylation sites does not affect the adipogenic potential of C/EBPβ (12), and the delay in DNA binding has since been questioned (13).

Interestingly, data suggest that increased proliferation capacity is associated with increased adipogenic potential. Mice lacking the two cyclin-dependent kinase inhibitors (CDKIs) p21 and p27 display adipose tissue hyperplasia (14). Also, MEFs lacking p21 undergo accelerated adipogenesis, although the CDKI was required for the survival of mature adipocytes (15).

The tumor suppressor p53 is critically involved in cell cycle control as evidenced by the massive and accelerated tumor development in p53-deficient mice (16). The p53 controls cell cycling through regulating expression of several genes, including p21. Here we show that MEFs lacking either p53 or p21 undergo spontaneous adipogenesis. The inhibitory effect of p53 on adipocyte differentiation relies on its transcriptional activity. Inhibition of adipocyte differentiation by forced expression of p53 or chemical blockage of mitotic clonal expansion was circumvented by the addition of a PPARγ agonist suggesting that the importance of mitotic clonal expansion is linked to a concomitant production of PPARγ agonist(s) necessary for the initiation of adipocyte differentiation.

MATERIALS AND METHODS

Plasmids

pCMVNeoBam-p53 and p55 R175D were generous gifts from Dr. Thierry Souissi. The p53 cDNAs were amplified using Prime-star (Takara) according to the manufacturer’s instructions, inserted into pBluescript, sequenced, and moved into pBABE-puro (a generous gift from Dr. Ormond A. MacDougald). pSicoRp53, pMD2.G, pMDLg/pRRE, and pRSV-Rev were purchased from Addgene; pSicoR-lacZ was a generous gift from Dr. Susanne Mani.

Cell culture, differentiation

Wild-type and p53-deficient MEFs were generous gifts from Dr. Stephen N. Jones and Dr. Guillermina Lozano. The p21-deficient and corresponding wild-type MEFs were kindly supplied by Dr. Jean-Christophe Marine. For spontaneous differentiation, MEFs were left confluent for 10 days with media (AmnioMax basal medium, Life Technologies Inc.) supplemented with 7.5% fetal bovine serum, 7.5% AmnioMax-C100 supplement, 2 mM glutamine, 62.5 µg/ml penicillin, and 100 µg/ml streptomycin, changed every second day. 3T3-L1 preadipocytes were grown and differentiated as described previously (17). Roscovitine (LC Laboratories), purvalanol (MERCK), paclitaxel (LC Laboratories), or rosiglitazone (kindly supplied by Novo Nordisk) were added to the media to the indicated concentrations throughout differentiation. For Oil Red O staining, cells were washed in PBS, fixed in 3.7% paraformaldehyde for 1 h, and stained as described (18).

Retro- and lentiviral transductions

Retroviral transduction was done as described previously with puromycin selection for 2 days (19). Lentiviral particles were produced as described (20). Lentiviral transduction was confirmed by green fluorescent protein expression.

Electroporation

3T3-L1 preadipocytes were electroporated with UASx4-TK-luc and GAL4-PPARγ-LBD 1 day past confluence according to the protocol described elsewhere (21). The day after electroporation, cells were treated as indicated, and the following day, cells were harvested and luciferase activity was measured according to the standard protocol.

Arachidonic acid release

Confluent 3T3-L1 preadipocytes were supplemented with radiolabeled arachidonic acid ([1-14C]arachidonic acid, Perkin Elmer) at an activity of 0.1 µCi/ml from day −2 to day 0. Before induction of differentiation, cells were rinsed three times with differentiation media. Cells were induced to differentiate as indicated. Two days past induction, cells were frozen to lyse cells and release intracellular free arachidonic acid. Cellular debris was removed by centrifugation. Scintillation fluid was added to the media, and radioactivity was determined.

RNA purification, reverse transcription, and real-time PCR

RNA was purified using TRIzol (Invitrogen) according to the manufacturer’s instructions. Reverse transcription was performed essentially as described elsewhere (8). Quantitative PCR (qPCR) was performed in 25 µl reactions containing SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma-Aldrich), 1.5 µl of diluted cDNA, and 300 nM of each primer. Reaction mixtures were preheated at 95°C for 2 min followed by 40 cycles of melting at 95°C for 15 s, annealing at 60°C for 30 s, and elongation at 72°C for 45 s. Primer sequences are available on request. Expression of TATA box binding protein was used for normalization.

Western blotting

Whole cell extracts were prepared as described previously (18). For the Western analyses, 100 µg of protein was loaded in each lane. After SDS-polyacrylamide gel electrophoresis, proteins were blotted onto polyvinylidene difluoride membranes (Micron Separation) using a Kem-En-Tec semidry blotter. Equal loading transfer was confirmed by Amidoblack staining of membranes. Membranes were blocked overnight in PBS containing 5% nonfat dry milk and 0.1% Tween 20 (Sigma). Incubation with primary and secondary antibodies was performed in PBS containing 5% nonfat dry milk for 1–2 h. After incubation with antibodies, membranes were washed in PBS containing 0.1% Tween 20. Secondary antibodies were horseradish peroxidase-conjugated anti-sheep or anti-rabbit antibodies (DAKO). Primary antibodies were sheep anti-p53 (Ab-7, MERCK), phospho-p53 antibody sampler kit (#9919, Cell Signaling), mouse anti-α-tubulin (DM1A, Sigma-Aldrich), and rabbit anti-TFIIB (C-18, Santa Cruz Biotechnology Inc.).

DAPI staining

3T3-L1 cells were induced as indicated followed by fixation and permeabilization by 90% methanol. Unspecific binding was blocked by replacing methanol with 5% skim milk in PBS. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen) in fresh blocking solution. Stained nuclei were scored by fluorescence microscopy.
EdU incorporation

The p53 $^{-/-}$ MEFs were cultivated in Amniomax, seeded in chamberslides, and 1 day after reaching confluence incubated with EdU (Click-iT® EdU Cell Proliferation Assay, Invitrogen) for 4 h. Four days later, cells were fixed with paraformaldehyde and permeabilized, and incorporated EdU was visualized by immunohistochemistry essentially as recommended by the manufacturer.

SYBR Green growth assay

3T3-L1 cells (grown in DMEM) or MEFs (grown in Amniomax) were transduced with empty vector (pBABE), p53wt, or p53 R175D mutant using standard protocols. After 3 days of selection with puromycin (3 μg/ml), cells were seeded in 48-well plates and induced to differentiate according to standard protocols. At the time of induction (day 0) and 4 days later (day 4), cells were harvested in 1% SDS; the amount of accumulated DNA was quantified by staining with SYBR Green, and fluorescence measured on an EnVision multi-label reader (PerkinElmer).

RESULTS

The understanding of adipocyte differentiation has been greatly facilitated by in vitro studies using MEFs and the established preadipocyte cell line 3T3-L1. The murine double minute 2 (mdm2) gene encodes the ubiquitin ligase Mdm2, which is a crucial regulator of p53 degradation. The mdm2 gene is amplified in 3T3-L1 cells (22), arguing that this cell line is less well suited for analysis of the effects of p53 on adipocyte differentiation and function. We therefore used MEFs to examine the impact of p53 on adipose conversion.

MEFs normally require a hormonal cocktail in order to induce adipose conversion. However, in contrast to wild-type MEFs, we observed that p53-deficient MEFs underwent spontaneous adipocyte differentiation (supplementary Fig. IA, B). Importantly, ectopic expression of p53 efficiently inhibited the spontaneous adipogenesis of p53-deficient MEFs (supplementary Fig. IC, D). Additionally, knockdown of p53 in wild-type MEFs leads to spontaneous formation of adipocytes (supplementary Fig. IE–G). Therefore, our data and those of others (23, 24) point to an inhibitory role for p53 in adipose conversion.

The p53 exerts both transcriptional and nontranscriptional effects. We sought to determine whether the inhibitory effect on adipogenesis relied on transcriptional activity of p53 by ectopic expression of either wild-type or a DNA binding-deficient mutant. Ectopic expression of wild-type p53 inhibited hormonally induced adipocyte differentiation of wild-type MEFs as determined by triglyceride staining with Oil Red O (Fig. 1A) and adipocyte marker gene expression (Fig. 1B). The inhibitory effect was dependent on the ability of p53 to bind to DNA, as a p53 mutant with

**Fig. 1.** Ectopic expression of p53 inhibits adipocyte differentiation of wild-type MEFs. Wild-type MEFs were transduced with either empty vector or vector encoding p53 or p53 R175D, selected, and differentiated. Eight days after induction, degree of differentiation was scored by triglyceride staining using Oil Red O staining (A) or adipocyte marker gene expression using real-time qPCR (B). * $P < 0.05$, one-way ANOVA. Error bars represent standard deviation. C: DNA content of transduced cells measured at days 0 and 4 by SYBR Green fluorescence analysis. * $P < 0.05$, one-way ANOVA. NS, nonsignificant. Error bars represent SEM. D: Western blot analyses of p53 and selected phosphorylated forms during adipocyte differentiation of wild-type MEFs. α-Tubulin was used as loading control.
impaired DNA binding ability (p53 R175D) (25) failed to inhibit adipose conversion of wild-type MEFs (Fig. 1A, B). Although p53 and p53 R175D were both expressed (supplementary Fig. IIA), only wild-type p53 induced expression of p21 (supplementary Fig. IIB) confirming the transcriptional inactivity of the p53 R175D mutant. Furthermore, wild-type p53 but not p53 R175D prevented cell division during the early stage of adipocyte differentiation as indicated by measurements of DNA content (Fig. 1C). The failure of p53 R175D to inhibit adipocyte differentiation strongly suggested that the transcriptional activity of p53 was required for its antiadipogenic effect.

To relate the possible inhibitory effect of p53 on hormonal induction of adipogenesis in wild-type cells, we analyzed the level of p53 and its phosphorylation status during the early stage of adipocyte differentiation of wild-type MEFs. Phosphorylation of p53 at numerous residues is reported to exert prominent control on the function of p53 (26). The total level of p53 and several of its N-terminal phosphorylations did not change over the course of adipose conversion (Fig. 1D). These phosphorylation events generally exert a stabilizing effect on p53 (26). However, phosphorylation of serine 389 (serine 392 in human) decreased during the first 2 days of differentiation and then later returned to starting levels (Fig. 1D). Interestingly, mutational analyses have shown that phosphorylation of this site is important for maintaining the basal level of expression of numerous p53 target genes (27, 28). It is possible that reduced phosphorylation at serine 389 of p53 promotes adipogenesis by reducing the transcriptional activity of p53 and thereby facilitates clonal expansion.

One of several cell cycle genes whose expression is perturbed by mutation of serine 389 is the CDKI p21 (28), one of the major players in p53-mediated cell cycle arrest (29). Expression of p21 was significantly lower in p53-deficient MEFs compared with wild-type MEFs at confluence (Fig. 2A). Lack of p21 has previously been associated with accelerated hormonally induced adipogenesis and hyperplasic adipose tissue (14, 15). We therefore speculated whether p21-deficient MEFs would recapitulate the spontaneous adipogenesis observed in p53-deficient MEFs. Indeed, maintaining wild-type and p21-deficient MEFs confluent for

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Fig. 2. The p21-deficient MEFs have increased adipogenic potential. A: Expression of p21 in confluent wild-type and p53-deficient MEFs as measured by real-time qPCR. * P < 0.05, Student’s t-test. Error bars represent standard deviation. B, C: Wild-type and p21-deficient MEFs were kept confluent for 10 days with fresh media added every second day. Adipose conversion was assessed by triglyceride staining using Oil Red O staining (B) or adipocyte maker gene expression using real-time qPCR (C). * P < 0.05, Student’s t-test. Error bars represent standard deviation. D, E: The p21-deficient MEFs were retrovirally transduced with either empty vector or vector encoding p53, selected, and kept confluent for 10 days with media changed every second day. Degree of adipogenesis was scored by adipocyte maker gene expression using Oil Red O staining (D) or adipocyte maker gene expression using real-time qPCR (E). * P < 0.05, Student’s t-test. Error bars represent standard deviation.
10 days with medium renewed every second day resulted in spontaneous adipose conversion of the p21-deficient MEFs, but not the corresponding wild-type MEFs as visualized by Oil Red O staining (Fig. 2B) and adipocyte marker gene expression (Fig. 2C). In keeping with the finding that p21 is required for survival of the mature adipocyte (15), we observed massive shedding of dead p21-deficient adipocytes to the medium (results not shown). Finally, supporting the notion that induction of p21 is necessary for the p53-dependent inhibition of adipocyte differentiation, forced expression of p53 did not inhibit spontaneous adipose conversion of p21-deficient MEFs (Fig. 2D, E).

The requirement for p21 and the DNA binding ability of p53 to inhibit adipogenesis suggested that continuous cell cycling might at least in part explain the spontaneous adipogenesis of p53-deficient MEFs. Confluent p53-deficient MEFs exhibited signs of increased cell cycling as they increased their DNA content relatively more than their confluent wild-type counterparts (Fig. 3A). To address whether the p53-deficient cells developing into adipocytes had undergone cell division/DNA replication, we pulse-labeled confluent p53-deficient with EdU and allowed the cells to differentiate spontaneously. More than 93% of labeled cells were adipocytes (Fig. 3B and data not shown).

To further link cell cycling to adipogenesis in p53−/− cells, we allowed these MEFs to undergo spontaneous adipocyte differentiation in the presence of vehicle or the chemical CDK1 roscovitine. Increasing concentrations of roscovitine led to a dose-dependent decrease in spontaneous adipose conversion of p53-deficient MEFs as seen by a decrease in the number of fat droplets (Fig. 3C) and adipocyte marker gene expression (Fig. 3D). Similar results were observed when treating p53-deficient MEFs with two other cell cycle inhibitors, paclitaxel and purvalanol (data not shown).

Concomitant with the mitotic clonal expansion, ligand(s) for PPARγ is generated. Interestingly, in vitro adipocyte differentiation of human cells occurs in the absence of mitotic clonal expansion but requires the addition of an exogenous PPARγ agonist (30). Adipocyte differentiation of C3H10T1/2 cells does not rely on completion of cell division (10). Efficient differentiation of these cells is, however, dependent on inclusion of a PPARγ agonist in the differentiation media unless pretreated with bone morphogenetic protein 4 or 7 (31–34). Of further interest is the observation that MEFs deficient for the retinoblastoma protein have irregular clonal expansion and require an exogenous PPARγ ligand in order to develop into adipocytes (18).

Accordingly, we speculated whether clonal expansion was necessary for PPARγ agonist production. To investigate this, we tested whether the inhibitory effect on adipogenesis of either synthetic cell cycle inhibitors or ectopic p53

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**Fig. 3.** Inhibition of cell cycling in p53-deficient MEFs abolishes their spontaneous adipogenesis. A: Relative increase in DNA amount in confluent wild-type and p53-deficient MEFs. DNA amount was measured by SYBR Green fluorescence and depicted as relative increase in DNA amount after 24 h compared with levels in confluent cells. *P < 0.05, Student’s t-test. Error bars represent SEM. B: Confluent p53-deficient MEFs were labeled with EdU for 4 h, rinsed, and kept confluent for 4 days with fresh media added at day 2. Incorporation of EdU and adipocyte differentiation was scored using microscopy. C, D: The p53-deficient MEFs were kept confluent for 10 days with fresh media added every second day in the presence of vehicle or increasing concentrations of roscovitine. Adipocyte differentiation was scored by triglyceride staining using Oil Red O staining (C) or adipocyte marker gene expression using real-time qPCR (D). Lowercase letters (a, b, c) denote different significance groups; P < 0.05, one-way ANOVA. Error bars represent standard deviation.
expression could be circumvented by addition of a PPARγ agonist. Indeed, rosiglitazone was able to restore adipocyte differentiation in 3T3-L1 cells treated with each of the cell cycle inhibitors as seen by Oil Red O staining (Fig. 4A) and, as exemplified by paclitaxel treatment, adipocyte marker gene expression (Fig. 4B). Along the same lines, rosiglitazone circumvented the inhibition of adipose conversion in wild-type MEFs mediated by forced expression of p53 (Fig. 4C, D), suggesting that the p53-mediated constraint of adipocyte differentiation precedes PPARγ agonist(s) production. In support of this, the augmented adipogenesis of p53-deficient MEFs was inhibited by addition of a PPARγ antagonist (24). Collectively, these results argue that mitotic clonal expansion is linked to the production of PPARγ agonist(s) during the initial stage of adipocyte conversion.

The generation of PPARγ ligand(s) during adipocyte differentiation has been verified using reporter assays measuring the activity of an isolated PPARγ-LBD (6, 8). We used a similar strategy to measure PPARγ ligand production in 3T3-L1 cells induced to differentiate in the presence or absence of cell cycle inhibitors. All inhibitors efficiently reduced PPARγ-LBD-mediated transactivation of the reporter gene (Fig. 5A) supporting the idea that inhibition of mitotic clonal expansion reduced the production of PPARγ agonist(s). In support of this, we found that the two inhibitors tested prevented the induction of the classic PPARγ target gene, aP2/ALBP (adipocyte lipid binding protein)/FABP4 (fatty acid binding protein 4), on day 1 following administration of the classic hormonal cocktail, whereas they did not prevent aP2/ALBP/FABP4 induction by rosiglitazone (Fig. 5B).

The nature of the true endogenous PPARγ agonist(s) inducing adipocyte differentiation remains enigmatic. It is, however, evident that the cAMP-elevating compound of the hormonal cocktail used to induce differentiation is responsible for initiating agonist production (6). Interestingly, elevated levels of cAMP were important for increasing the number of cells in the differentiating 3T3-L1 cells (Fig. 5C).

We have recently suggested that hepoxilins act as agonists during the initial stages of adipogenesis. The generation of hepoxilins during adipogenesis is likely to rely on a reactive oxygen species (ROS)-mediated conversion of arachidonic acid or structurally similar PUFAs into substrates for the unconventional lipoxygenase, epidermal type-3 lipoxygenase (eLOX3) (35). The source of these PUFAs was, however, not determined. As cAMP is suggested to be the priming factor for PPARγ agonist production, we speculated whether cAMP could be linked to the release of PUFAs from phospholipids in cellular membranes. Therefore, we attempted to estimate free, endogenous arachidonic acid in 3T3-L1 cells treated with dexamethasone, 3-isobutyl-1-methylxanthine (MIX), or insulin. In short, 3T3-L1 cells were grown in media containing 14C-labeled arachidonic acid. Before stimulation, the labeled cells were rinsed thoroughly. Intracellular free arachidonic acid was released to media by freeze-thaw lysing of the cells. Before assaying radioactivity in cell lysate, debris was spun down. Although this assay only led to a rough estimate of released arachidonic acid, only the cAMP-elevating compound MIX tended to increase the intracellular free pool of arachidonic acid (Fig. 6A).

As our results suggested that mitotic clonal expansion is intimately associated with ligand production, we sought to...
DISCUSSION

During recent years, there has been an explosion in knowledge of adipocyte differentiation. Still, the role of mitotic clonal expansion has been a matter of dispute. Here we provide data arguing that mitotic clonal expansion is important for the concurrent PPARγ ligand production. We demonstrate that an exogenous PPARγ agonist was capable of restoring adipogenesis in cells where cell cycling was ablated either chemically or genetically. Furthermore, the chemical inhibitors of cell cycling prevented the activation of a PPARγ-responsive reporter plasmid.

As discussed previously, mitotic clonal expansion has been suggested to be required for activation of C/EBPβ (11). Recently, induction of xanthine oxidoreductase (XOR) expression during adipose conversion was shown to be highly dependent on C/EBPβ (36). As XOR expression can be used as a measure for C/EBPβ activity, we quantified induction of XOR in differentiating 3T3-L1 cells treated with vehicle or cell cycle inhibitors. Although the inhibitors blocked MDI-induced adipose conversion (Fig. 4A, B), they did not affect the induction of either C/EBPβ or XOR during the early stages of adipocyte differentiation (Fig. 6C) suggesting that the requirement for mitotic clonal expansion in adipogenesis is not related to expression or activation of C/EBPβ.
Although the mechanism was not described, Farmer and colleagues pinpointed C/EBPβ as an essential regulator of ligand production (41). A later study by the laboratory of Friedman strongly suggested that C/EBPβ acted through induction of xanthine XOR expression (36). Still, the exact mechanism by which XOR augmented PPARγ activity remained unresolved.

We have then proposed that lipid autooxidation by ROS generated by XOR results in the production of substrates for eLOX3. eLOX3 converts the peroxidized lipids into hepoxilin-like products that function as endogenous PPARγ agonists during adipose conversion (35). The dependency on eLOX3 in PPARγ ligand production is consistent with the observation that a lipoxygenase inhibitor, baicalein, decreases PPARγ ligand generation (8). However, the source of lipids for the autooxidation reaction was unknown.

eLOX3 can convert peroxidized fatty acids originating from arachidonic and linoleic acid (42, 43). These fatty acids are present in phospholipids embedded in cellular membranes. Their release is mainly regulated by the actions of phospholipases. Of the known families of phospholipases, PLA2, PLC, and PLD, only PLA2 releases the free fatty acid directly. Interestingly, the activity of cytoplasmic phospholipase A2 (cPLA2) is upregulated by proliferation (44). The activity and localization of cPLA2 are regulated by an activating phosphorylation by p42/p44 MAPKs. This phosphorylation leads to localization of cPLA2 to the nuclear membrane (45).

cAMP is responsible for the activation of MAPK early during adipogenesis (46). Based on our recent findings, we hypothesize that cAMP elevates MAPK activity through activation of the exchange proteins directly activated by cAMP (17). Through its activation of MAPK, the second messenger may thereby lead to the redistribution of cPLA2 to the nucleus. It is therefore possible that the extensive remodeling of the nuclear membrane during the clonal expansion is a prerequisite for efficient PUFA release by cPLA2.

Besides the suggested pathway, it is possible that the elevated release in arachidonic acid reflected an increase in the release of nitrolinoleic acids. These fatty acids are reported to be potent PPARγ ligands (47). However, this does not fit our observation that inhibition of lipoxygenase activity decreases PPARγ ligand generation during adipocyte differentiation (8). Still, it is possible that nitrolinoleic acids contribute to the PPARγ ligand pool under adipose conversion.

Cancer cells are defined by their uncontrolled proliferation. Furthermore, these cells are known to have high levels of ROS rendering them capable of producing PPARγ agonists by the mechanism described previously. The plausible increased generation of PPARγ agonists in cancers would seem counterintuitive considering the antiproliferative effect of PPARγ and its ligands (48). However, because PPARγ is mutated in several tumors and tumor-derived cells lines (49–51), it is possible that inactivation of PPARγ can circumvent the antiproliferative effects of the PPARγ ligands generated by increased proliferation and ROS levels.

In conclusion, our results provide evidence for a cell cycle-dependent production of endogenous PPARγ ligands necessary for initiation of adipocyte differentiation and with possible implications in relation to other cell types with deregulated cell cycle control.

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