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PPARγ1 Synthesis and Adipogenesis in C3H10T1/2 Cells Depends on S-Phase Progression, but Does not Require Mitotic Clonal Expansion

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Abstract Adipogenesis is typically stimulated in mouse embryo fibroblast (MEF) lines by a standard hormonal combination of insulin (I), dexamethasone (D), and methylisobutylxanthine (M), administered with a fresh serum renewal. In C3H10T1/2 (10T1/2) cells, peroxisome proliferator-activated receptor γ1 (PPARγ1) expression, an early phase key adipogenic regulator, is optimal after 36 h of IDM stimulation. Although previous studies provide evidence that mitotic clonal expansion of 3T3-L1 cells is essential for adipogenesis, we show, here, that 10T1/2 cells do not require mitotic clonal expansion, but depend on cell cycle progression through S-phase to commit to adipocyte differentiation. Exclusion of two major mitogenic stimuli (DM without insulin and fresh serum renewal) from standard IDM protocol removed mitotic clonal expansion, but sustained equivalent PPARγ1 synthesis and lipogenesis. Different S-phase inhibitors (aphidicolin, hydroxyurea, L-mimosine, and roscovitin) each arrested cells in S-phase, under hormonal stimulation, and completely blocked PPARγ1 synthesis and lipogenesis. However, G2/M inhibitors effected G2/M accumulation of IDM stimulated cells and prevented mitosis, but fully sustained PPARγ1 synthesis and lipogenesis. DM stimulation with or without fresh serum renewal elevated DNA synthesis in a proportion of cells (measured by BrdU labeling) and accumulation of cell cycle progression in G2/M-phase without complete mitosis. By contrast, standard IDM treatments with fresh serum renewal caused elevated DNA synthesis and mitotic clonal expansion while achieved equivalent level of adipogenesis. At most, one-half of the 10T1/2 mixed cell population differentiated to mature adipocytes, even when clonally isolated. PPARγ was exclusively expressed in the cells that contained lipid droplets. IDM stimulated comparable PPARγ1 synthesis and lipogenesis in isolated cells at low cell density (LD) culture, but in about half of the cells and with sensitivity to G1/S, but not G2/M inhibitors. Importantly, growth arrest occurred in all differentiating cells, while continuous mitotic clonal expansion occurred in non-differentiating cells. Irrespective of confluence level, 10T1/2 cells differentiate after progression through S-phase, where adipogenic commitment induced by IDM stimulation is a prerequisite for PPARγ synthesis and subsequent adipocyte differentiation. J. Cell. Biochem. 91: 336–353, 2004.
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Key words: adipogenesis; PPARγ; DNA synthesis; mitotic clonal expansion; cell cycle

The conversion of mouse embryo fibroblasts (MEF) to adipocytes, following hormonal stimulation, has been extensively studied as a means of identifying key regulatory factors involved in adipocyte differentiation. Most studies have used the preadipocyte cell line, 3T3-L1, which is stimulated to differentiate, in vitro, by a hormonal mixture (IDM) consisting of insulin (Ins, I), the glucocorticoid dexamethasone (DEX, D), and the phosphodiesterase inhibitor methylisobutylxanthine (MIX, M) [Green and Meuth, 1974; Green and Kehinde, 1975]. This same hormonal mixture induces differentiation of immortalized embryo fibroblast lines, including C3H10T1/2 (10T1/2), and also primary embryo fibroblasts [Alexander et al., 1998]. Hormonal induction of these embryo fibroblasts typically forms a substantially lower proportion of adipocytes than 3T3-L1 cells. The MEFs are pluripotent and differentiate to various mesenchymal cell lineages under appropriate stimulation [Taylor and Jones, 1979; Konieczny

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and Emerson, 1984]. The process of adipogenesis can be initiated in otherwise resistant fibroblasts, such as the NIH3T3 cell line, by transfection of a series of the key adipogenic nuclear factors that are expressed or activated in response to IDM-stimulation, including CCAAT/enhancer binding protein β (C/EBPβ), PPARγ, C/EBPα, and adipocyte determination and differentiation dependent factor 1/sterol regulatory element-binding protein 1(ADD1/SREBP1) [Chawla and Lazar, 1994; Wu et al., 1995; Kim and Spiegelman, 1996; Shao and Lazar, 1997]. This has lead to a general hypothesis that the hormonal mixture stimulates a cascade involving these key adipogenic regulators, which then directs cell cycle exit and the insulin-stimulated conversion of glucose to triglycerides.

Adipocyte differentiation of 10T1/2 cells, in vitro, by hormonal stimulation divides into two phases, an initial 48 h period in which a cascade of steps leads to cell cycle exit and a subsequent period of several days in which insulin stimulates lipogenic genes [Gregoire et al., 1998]. Dramatic elevation of PPARγ and then C/EBPα expression immediately precedes the cell cycle exit [Gregoire et al., 1998] and mediates growth arrest [Umek et al., 1991; Timchenko et al., 1996]. Peroxisome proliferator-activated receptor γ1 (PPARγ1) directly mediates the increase of C/EBPα protein in the early phase of adipogenic induction [Wu et al., 1999]. The p42 isoform of C/EBPα mediates this and other transcriptional activities, whereas the proteolytic product, p30, inhibits this function [Umek et al., 1991]. Growth arrest of 3T3-L1 cells is associated with increased levels of several cyclin-dependent kinase inhibitors, including $p18^{INK4C}$, $p21^{Waf1/Cip1}$, and $p27^{Kip1}$, which may be driven by increased PPARγ expression [Morrison and Farmer, 1999]. PPARγ-mediated loss of DNA binding activity of E2F/DP has also been linked to growth arrest [Altiok et al., 1997]. The sequence of gene expression changes in 10T1/2 cells is very similar to the sequence seen in the more rapidly and extensively responding 3T3-L1 cells [Hamm et al., 2001].

Cell cycle exit during the first stage of hormonal stimulation is followed by the elevated expression of genes that mediate the conversion of glucose and fatty acids to triglycerides, which characterizes the mature adipocytes [Timchenko et al., 1996; Altiok et al., 1997; Morrison and Farmer, 1999]. Adipogenesis is stimulated by PPARγ ligands, such as the thiazolidinedione, BRL49653 (BRL). The effect of BRL is more substantial in 10T1/2 cells than in 3T3-L1 cells [Schluman et al., 1998]. PPARγ1 and C/EBPα, along with ADD1/SREBP1, mediate the delayed formation of PPARγ2 and, together, these factors mediate the insulin transcription of lipogenic genes [Christy et al., 1989; Umek et al., 1991; Freytag et al., 1994; Tontonoz et al., 1994a,b; Timchenko et al., 1996]. PPARγ becomes transcriptionally active by forming a heterodimer with retinoid X receptor (RXR), which also mediates enhanced adipogenesis through ligand activation [Lehmann et al., 1995; DiRenzo et al., 1997].

The standard hormonal stimulation by IDM used in most studies is accompanied by a reintroduction of fresh fetal bovine serum (FBS) [Green and Meuth, 1974; Green and Kehinde, 1975], which produces a rapid and extensive activation of growth factor receptors and downstream kinase cascades, including the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI 3K) pathways [Párrizas et al., 1997; Whithers and White, 2000]. Insulin also provides a mitogenic stimulus [Qiu et al., 2001]. Together, these stimuli cause a mitotic clonal expansion of the confluent 3T3-L1 preadipocytes [Qiu et al., 2001]. Several studies of 3T3-L1 cells have provided evidence that extracellular signal regulated kinase (ERK) activation and mitotic clonal expansion are essential to adipocyte differentiation [Font de Mora et al., 1997; Kim et al., 2001]. However, a recent report provides evidence that comparable adipogenesis can be induced, without mitotic clonal expansion, when 3T3-L1 cells are stimulated with hormonal mixture that does not include insulin [Qiu et al., 2001]. Recent studies [Qiu et al., 2001] further suggest that mitotic clonal expansion is not necessary in 3T3-L1 sublines, which provide a more rapid response to IDM stimulation, paralleling the response in 10T1/2 cells.

In the present study, we have examined whether mitotic clonal expansion is essential for adipocyte differentiation of 10T1/2 cells. We define a set of non-mitogenic conditions that are fully effective for adipogenic stimulation of 10T1/2 cells in the absence of cell division. The use of various protocols for adipogenic induction demonstrates that PPARγ expression and lipogenesis are restricted to the same fraction of

10T1/2 cells, but that this differentiation does not correlate with extent of DNA synthesis. More specifically, we use selective cell cycle inhibitors to establish that some level of DNA synthesis and progression into S-phase is essential for PPARγ induction and lipogenesis, whereas mitosis is not necessary. We also show that low density (LD) 10T1/2 cultures exhibit similar differentiation characteristics and that, notably, only non-differentiating cells exhibit continuous clonal expansion.

MATERIALS AND METHODS

Materials

Anti-PPARγ, anti-C/EBPβ, anti-C/EBPα, and anti-SREBP1 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibodies were purchased from Promega (Madison, WI). FITC- and Rhodamine-conjugated secondary antibodies were purchased from Molecular Probes, Inc. (Eugene, OR). Nitrocellulose membranes and ECL detection kit were purchased from Amersham Biosciences (Piscataway, NJ). G1/S inhibitors (aphidicolin, hydroxyurea, L-mimosine, and roscovitin) and G2/M inhibitors (nocodazole, paclitaxel, colchicine, and colcemide) were purchased from Sigma Chemical (St. Louis, MO). Anti-BrdU antibody was provided by Dr. S. Swaminathan, (University of Wisconsin, Madison, WI).

Cell Culture and Differentiation Induction of 10T1/2 MEFs

The 10T1/2 MEFs (ATCC, Bethesda, MD) were cultured in Dulbecco's modified Eagle's medium:F-12 nutrient mix (DMEM:F12, Gibco/Invitrogen, Carlsbad, CA), supplemented with 10% FBS (Atlanta Biologicals, Norcross, GA), 100 U/ml of penicillin, and 100 μg/ml streptomycin (Gibco/Invitrogen). Cultured cells were maintained at 37°C in a humidified 5% CO_2 atmosphere. Differentiation of 2-day post-confluent cells by the standard protocol was initiated with 10 μg/ml insulin (I), 1 μM dexamethasone (D), 0.5 mM methylisobutylxanthine (M) in DMEM:F12 supplemented with 10% FBS (designated as day 0). After 48 h (day 2) incubation, the culture medium was replaced with DMEM:F12 supplemented with 10% FBS and 10 μg/ml insulin. In an alternate protocol, DM or IDM mixtures in DMEM:F12 medium were added to the post-confluent cells without change of medium and serum (unrenewed serum (URS)). To generate conditioned medium equivalent to that used in the URS protocol, DMEM:F12 medium supplemented with fresh FBS was incubated with 90% confluent cells for 2 days and collected.

For induction of adipogenesis in LD cultures, equal numbers of 10T1/2 cells from confluent cultures were initially dispersed at approximately 2–5% of the confluent density, such that cell–cell contacts were minimal even after 2 days of culture. LD cells were stimulated with IDM or DM for 4 days before the culture medium was replaced with DMEM:F12, supplemented with 10% FBS and 10 μg/ml insulin. This medium was replaced every 2 days.

For cell cycle inhibitor treatments, G1/S inhibitors (10 μM aphidicolin, 3 mM hydroxyurea, 100 μM L-mimosine, or 25 μM roscovitin) or G2/M inhibitors (2.5 μg/ml nocodazole, 1 μM paclitaxel, 100 nM colchicines, or 1 μM colcemide) were added to the cells with the respective hormone mixture on day 0. G1/S inhibitors were replaced with preconditioned medium obtained from cell culture dishes, as described above.

Cell Counting and Fluorescence Activated Cell Sorting (FACS) Analysis

10T1/2 cells from 5-cm dishes (confluent cells) or from 15-cm dishes (LD cells) were collected through trypsination by centrifugation. For cell counting, an aliquot of collected cells was counted using a hemacytometer plate. The cells were then fixed in 70% ethanol, treated with 0.5 mg/ml RNase A for 1 h at room temperature, and subsequently stained with 20 μg/ml propidium iodide overnight. The DNA content was determined by FACS analysis using a CELL FIT software package.

Oil-Red-O Staining

For Oil-Red-O staining of the lipid droplets, 10T1/2 cells, at day 8, were washed three-times with PBS and then fixed for 5 min with 4% paraformaldehyde (PFA). Fixed cells were incubated with diluted Oil-Red-O (2.1 mg/ml [4 isopropanol:3 water]) for 1 h at room temperature. Stained lipid droplets in cell monolayers were visualized by light microscopy and photographed.

Immunofluorescence and BrdU Labeling

10T1/2 cells were plated onto sterile glass coverslips in 6-well plates and cultured until

post-confluence. These cells were then induced to differentiate, as indicated above. For immunofluorescence at day 2 and 4 after induction, cell monolayers were fixed with 4% PFA, permeabilized with 0.05% TritonX-100 in PBS, and then blocked with 2% bovine serum albumin (BSA) and 3% goat serum (GS) in PBS for 45 min at room temperature. Cells then were incubated with the primary antibody (anti-PPARγ antibody, 1:500 dilution) in 2% BSA and 3% GS for 1 h at room temperature, followed by incubation with FITC-conjugated secondary antibody for 1 h at room temperature. After each step, cells were washed with PBS five-times. The cover slips were mounted with antifade solution (Molecular Probes, Inc.) for subsequent immunofluorescence microscope analysis (AXIOSCOP 20; Zeiss, Hallbergmoos, Germany). For BrdU labeling, post-confluent cells were induced to differentiate with appropriate hormonal stimuations. At designated time points after hormonal stimulation, 30 μg/ml BrdU was added for 3 h, and the labeling medium was replaced with preconditioned medium, as described previously. On day 4, coverslips were fixed in 70% ethanol for 30 min. Fixed cells on the coverslips were then treated with 1.5 M HCl, permeabilized with 0.05% TritonX-100 in PBS, and then blocked with 2% BSA and 3% GS in PBS for 45 min at room temperature. Blocked cells were incubated with anti-BrdU primary antibody (1:200 dilution) for 1 h at room temperature. After washing, FITC-conjugated secondary antibody (1:200 dilution) containing 0.1 μg/ml DAPI was added to the coverslips for 1 h at room temperature. Mounting and analyses were as described above.

Preparation of Total Cell Lysates and Immunoblot Analysis

After adipogenic stimulation (for the designated time period), cell monolayers, were washed three-times with ice-cold PBS and then scraped into RIPA lysis buffer (150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 1% NP-40, 0.1% SDS, 50 mM Tris Base pH 7.4, 0.25% deoxycholate, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotonin). Cell lysates were heated at 95°C for 5 min. The 50 μg of total protein, determined by Pierce BCA Kit (Rockford, IL) according to the manunufacturer's protocol, was separated by 7.5% (for PPARγ and ADD1/SREBP1) and 10% (for C/EBPα and C/EBPβ) SDS–PAGE, and then transferred to a nitrocellulose membrane. Target proteins were detected by horseradish peroxidase-congugated secondary antibodies and visualized with ECL^{TM} Western blotting detection reagents (Amersham Biosciences).

RESULTS

DM Stimulation Induces C/EBPβ and C/EBPα in Parallel With PPARγ. Insulin and Serum Renewal Have Little Effect on PPARγ1 Expression

In the standard differentiation protocol, insulin (I) is added with DEX (D) and MIX (M). These three stimulants of the standard hormonal mixture (IDM) are typically added in new medium supplemented with fresh serum. In this standard protocol, insulin and fresh serum each cause mitotic clonal expansion [Párrizas et al., 1997; Whithers and White, 2000].

In our present study, we have first examined adipocyte differentiation in 10T1/2 cells with respect to the contribution of individual components of the standard hormonal mixture. In particular, we have examined whether the mitogenic stimuli provided by insulin or fresh serum are essential for the synthesis of key adipogenic regulators, including C/EBPβ, PPARγ, and C/EBPα. We analyzed the effects of each hormonal stimulus on the expression of these key adipogenic regulators (Fig. 1A). MIX alone was sufficient to induce C/EBPβ expression, which was not affected by DEX or insulin, either singly or in combination. The sensitivity of C/EBPβ induction to the cAMP signal provided by MIX parallels previous observations with 3T3-L1 cells [Cao et al., 1991]. By contrast, the combined stimulation of DEX and MIX (DM) was essential and sufficient for optimal induction of PPARγ and C/EBPα, although insulin had only a slight effect (Fig. 1A).

DM sequentially stimulated C/EBPβ, PPARγ, and C/EBPα expression (Fig. 1B). C/EBPβ expression (peaked after 6 h) reached the highest level prior to elevation of PPARγ expression (increased 24–36 h), while elevation of C/EBPα expression only appeared after 36 h of stimulation. Although insulin only modestly increased the PPARγ1 expression at the 48 h period, its presence advanced the stimulation by approximately 6 h (Fig. 1B). The presence or absence of an initial fresh serum renewal had no effect on either PPARγ or C/EBPα expression (Fig. 1C).

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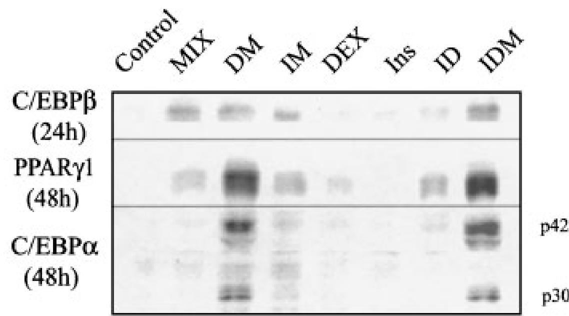

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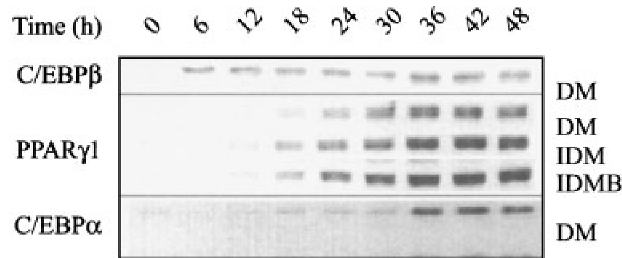

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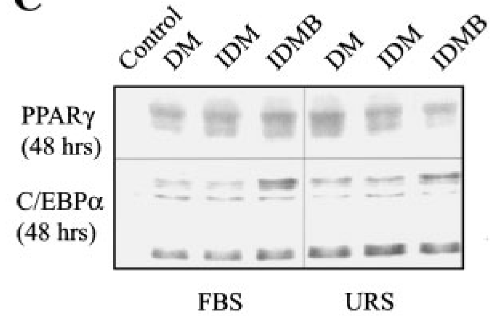

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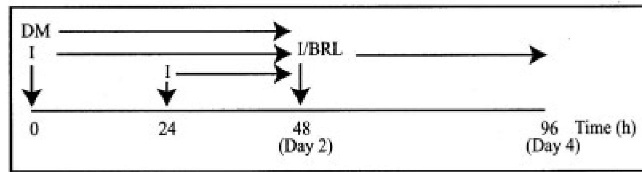

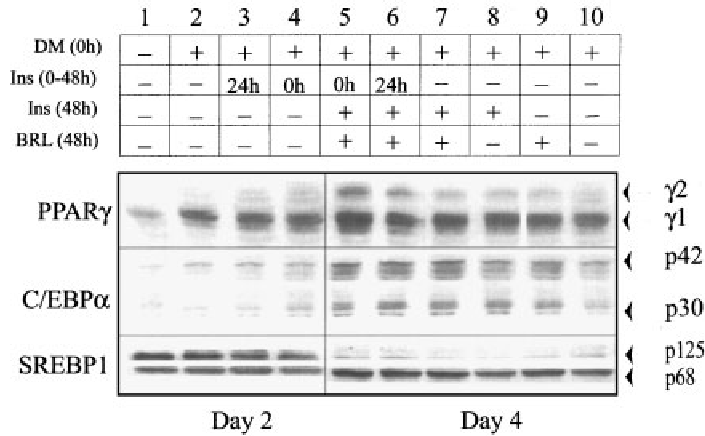

Fig. 1. Insulin and serum renewal have little effect on peroxisome proliferator-activated receptor γ1 (PPARγ1) expression. **A**: Effects of hormonal components. Post confluent C3H10T1/2 (10T1/2) cells were treated with each of the hormonal components individually (DEX, MIX, insulin (Ins)), in various combinations (DEX/MIX (DM), insulin/MIX (IM), insulin/DEX (ID), insulin/DEX/MIX (IDM)), or without additions (control) along with FBS renewal. Treated cells were harvested either 24 or 48 h after treatment. Total cell lysates (50 μg) were subjected to Western blot analysis using the designated antibodies (also for **B–D**). The Western blot shown is representative of three independent experiments. B: Time course. Cells were treated with DM or IDM and then analyzed at the designated time points. C: Effects of initial serum renewal. Cells were treated with DM, IDM, or IDMB (Insulin/DEX/MIX/BRL49653 (BRL)) in medium supplemented with fresh FBS or without serum renewal (unrenewed serum (URS)). The blot shown is representative of three independent experiments. D: Effects of insulin and BRL. Insulin was added at the indicated times (0 or 24 h) to cells, treated with DM with fresh FBS, and were analyzed after 48 h (day 2). DM-treated cells were further treated after 48 h with new medium containing fresh FBS, with or without addition of insulin and/or BRL, and then were analyzed 2 days later (day 4). The Western blot shown is representative of two independent experiments.

Early Insulin Addition Accelerates PPARγ2 Expression

We next tested whether omission of insulin from adipogenic hormonal mixture exerts further early effects on adipogenesis. Previous study on 3T3-L1 cells has suggested that insulin may, additionally, stimulate the activity of PPARγ1 [Tontonoz et al., 1994b]. We tested this hypothesis in 10T1/2 cells through addition of insulin at different time points after initiation of DM stimulation (Fig. 1D). The presence of insulin throughout the initial 48 h stimulation period (standard protocol, compare columns 5–7) progressively increased PPARγ2 expression, which occurred after PPARγ1 and was particularly evident at day 4. These insulin additions did not affect either PPARγ1 or C/EBPα expression, particularly during the 48–96 h period (day 2–4).

The PPARγ agonist, BRL, doubled the C/EBPα expression level when added at day 2 (after DM removal), consistent with the role of PPARγ in this step [Hamm et al., 2001] (Fig. 1D). Insulin and BRL were equally effective in stimulating PPARγ2 and C/EBPα expression, but were not additive (compare columns 7–10). Insulin can apparently replace BRL in providing activation of PPARγ1 in this second stage.

Proteolytic cleavage of SREBP1 (p125) to the functionally active p68 nuclear regulator also increased between day 2 and 4, and was modestly increased by the initial presence of insulin (Fig. 1D). This may contribute to the delayed stimulation of PPARγ2, which, unlike PPARγ1, has SREBP1 responsive elements in the promoter region [Kim et al., 1998].

PPARγ Is Exclusively Expressed in Lipogenic Cells

We have used culture conditions that effect the lipogenic conversion of 10T1/2 cells in order to test the linkage between PPARγ expression and lipogenesis in individual cells. At most, 50% of the 10T1/2 cells converted to mature adipocytes when stimulated with the IDM or DM hormonal mixtures. Adipogenic conversion by IDM stimulation was analyzed using several clonally isolated sub-line cells to minimize variables of genetic heterogeneity within the mixed population of 10T1/2 cell line. The extent of adipogenic conversion of three sub-clonal lines was, in each case, very similar and also no more extensive than for the heterogeneous parental 10T1/2 cells (Fig. 2A). This partial adipogenic conversion of 10T1/2 cells is, therefore, not determined by genetic differences, but, rather, is an inherent property of the multipotent cells. We determined whether PPARγ expression occurred in all of the stimulated cells that generated lipid droplets. The IDM-stimulated adipocytes (day 8) were stained immunohistochemically with anti-PPARγ antibody and the same field was subsequently examined for lipid droplets by phase contrast microscopy. PPARγ was only expressed in approximately half of the cells and was exclusive to lipogenic cells (Fig. 2B). The remaining cells were resistant to the adipogenic stimulation. The proportion of cells that expressed PPARγ and formed lipid droplets was relatively insensitive to either serum renewal or insulin addition (Fig. 3A,B). Similarly, 50% of cells expressed PPARγ at day 2 and 4 prior to formation of lipid droplets (data not shown). These experiments clearly show that the linkage between PPARγ expression, lipogenesis, and a proportion of the adipogenesis-inducible cells was independent of the mitogenic stimulation.

DM Stimulation Causes S-phase Progression and Adipogenesis, but Does not Lead to Mitosis

Previous study has implicated the post-confluent mitotic clonal expansion as an essential step for adipogenesis in 3T3-L1 cells [Tang et al., 2003]. The correlation of PPARγ expression and lipogenesis in 10T1/2 cells, after exclusion of the prime mitogens, suggested that this is not the case for 10T1/2 cells. We confirmed that insulin, in conjunction of fresh serum renewal, was the major mitogen for these confluent cells (70% increase in cell number), while FBS alone caused a modest 20% increase of cell number after 48 h (Fig. 4A). DM did not affect the mitogenic stimulus of insulin when accompanied by serum renewal. However, serum renewal was essential for the mitogenic effect of IDM stimulation. This data points to a mitotic synergism between insulin and serum factors that is unopposed by DM. The effectiveness of DM as a stimulant of PPARγ expression and lipogenesis indicates that these processes occur in 10T1/2 cells without mitotic clonal expansion.

We also measured de novo DNA synthesis in individual cells by means of BrdU labeling. IDM-stimulated DNA synthesis was visible in a

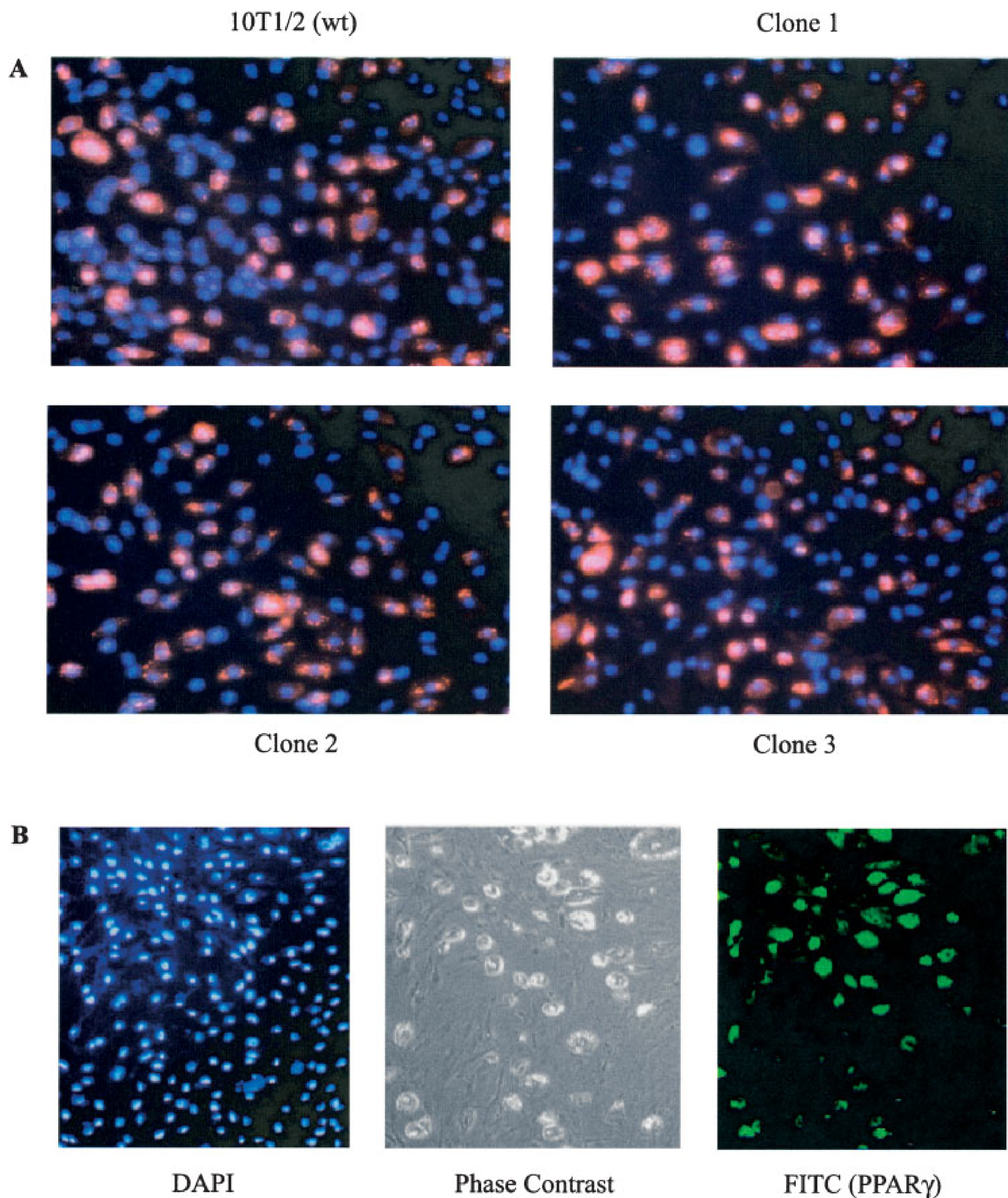

Fig. 2. PPARγ is selectively expressed in lipogenic cells. **A**: Adipogenesis at day 8 in four sub-lines. Proliferating cells (parental and clonally isolated sub-lines) were cultured on glass coverslips until confluence and then (day 0) treated with IDM in FBS-supplemented medium. After 48 h of treatment, cells were cultured in medium containing insulin (replenished every 2 days upto day 8). Treated cells were stained with Nile-Red (Red) and DAPI (blue) for the visualization of cytoplasmic triglyceride droplets, and nuclei, respectively, using fluorescence microscopy. **B**: Immunofluorescence analysis of PPARγ expression and lipid droplets in adipocytes (at day 8), generated as described in (A). From left to right: DAPI-stained for nuclei; phase-contrast image for lipid droplets; FITC-labeled anti-PPARγ antibody. The microscopes show representative fields of each treatment group for two experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

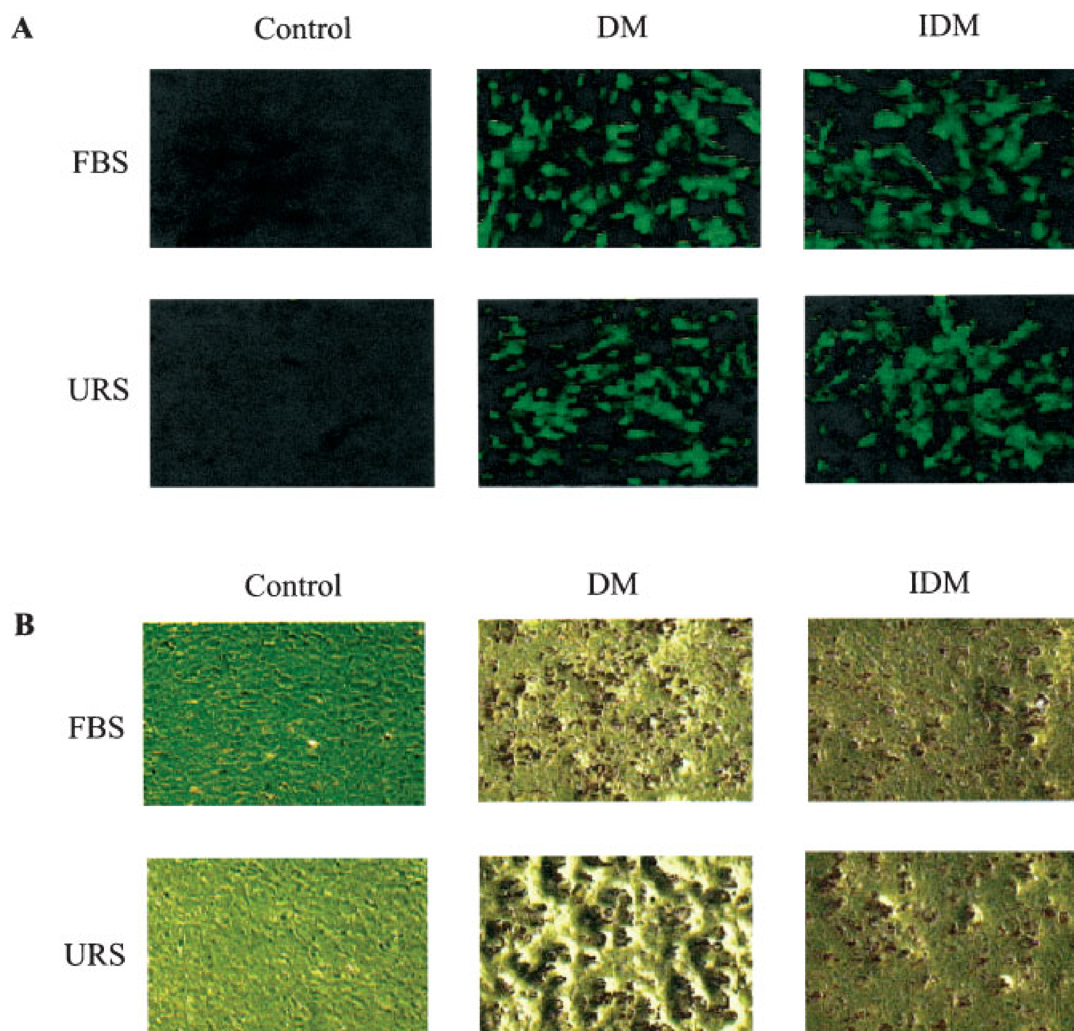

Fig. 3. DM stimulation optimally induces PPARγ expression and lipogenesis, irrespective of serum renewal. **A**: PPARγ expression. Post-confluent cells treated with hormonal mixture in FBS-supplemented medium (DM/FBS or IDM/FBS) or unrenewed serum (DM/URS or IDM/URS) were immunohistochemically stained for PPARγ expression at day 2 and visualized by immunofluorescence microscopy. The microscopes show representative fields of each treatment group for three experiments. **B**: Lipogenesis. Cells treated in this way were stained at day 8 with Oil-Red-O reveal cytoplasmic triglyceride droplets and were examined by phase contrast microscopy. The microscopes show representative fields of each treatment group for three or more experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

limited number of cells during a period of 9–12 h after stimulation, increased greatly during the 12–15 h period, and then declined substantially for the subsequent three labeling periods (Fig. 4B). This stimulation of DNA synthesis was detectable in less than half the cells. We have used the 12–15 h period to compare the effects of insulin and serum renewal (Fig. 4C). DM stimulation substantially elevated DNA replication, but there was no complete mitosis followed up resulting in no mitotic clonal expansion. The DNA synthesis induced by DM stimulation was elevated cooperatively by both insulin and serum renewal. Level of DNA synthesis by hormonal stimulation varied between individual cells. Although the proportion of stimulated cells with elevated DNA synthesis was less than for PPARγ expression and lipogenesis, it does not indicate that DNA synthesis is not required step for adipogenesis [Qiu et al., 2001]. We examined that inhibition of DNA synthesis completely blocked adipogenesis (Fig. 5). The standard mitogenic IDM/FBS stimulation produced only a modest increase in DNA synthesis over non-mitogenic DM/FBS or DM/URS stimulation.

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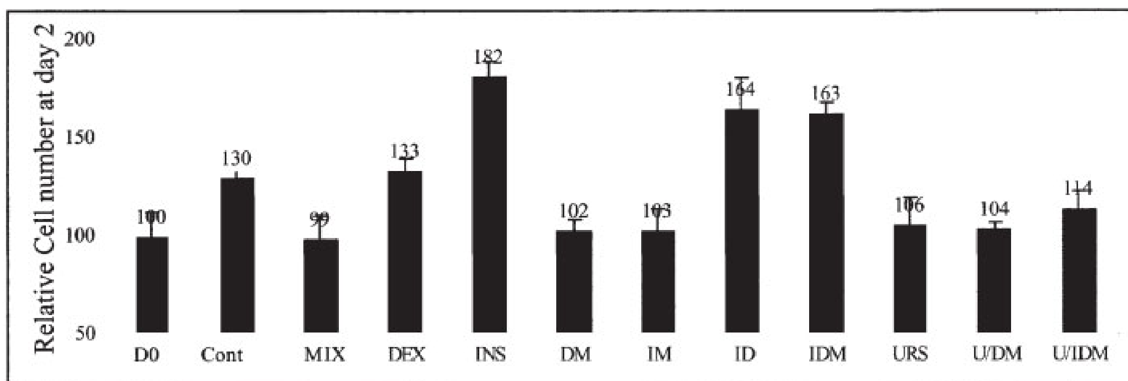

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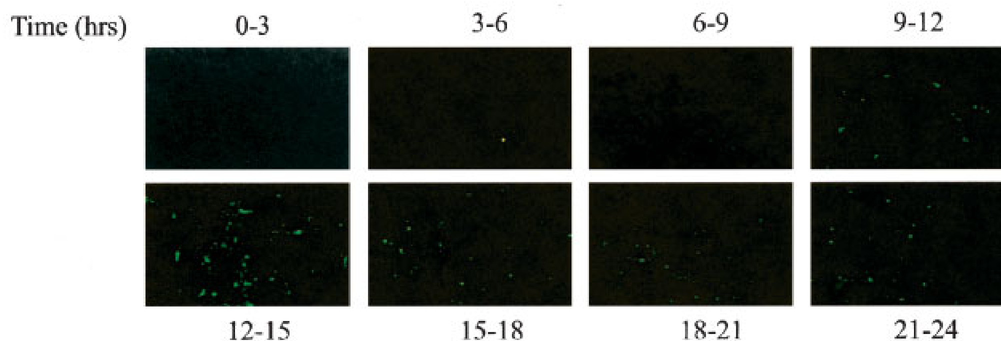

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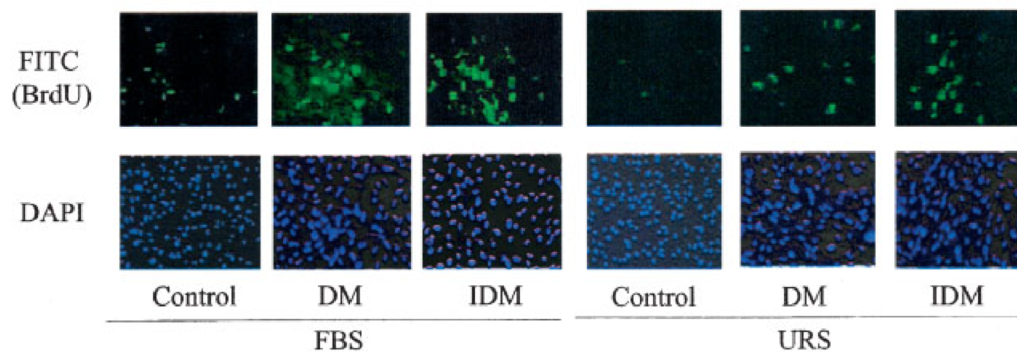

Fig. 4. Mitosis and the level of DNA synthesis are not proportionally linked to adipogenesis. **A**: Mitotic clonal expansion. Post-confluent cells were treated with each hormonal component individually or in combination for 48 h, as in Figure 1A. Cell numbers were determined at day 2 after hormonal stimulation. Experiments were done in triplicate with two different times. The average cell number at day zero (day 0) is normalized to 100. Data are presented as average ± SD. **B**: Immunofluorescence analysis of BrdU incorporation by time course. Post-confluent 10T1/2 cells were treated for 48 h with IDM mixtures, as described in Figure 1B, under the two different serum conditions. BrdU was pulsed for 3 h at the indicated time intervals. Cells containing BrdU labeled DNA with FITC-labeled anti-BrdU antibodies were visualized by immunofluorescence microscopy. The microscopes show representative fields of each treatment for two independent experiments. **C**: BrdU was pulsed from the 12–15 h following IDM treatment (the period of highest BrdU incorporation), and visualized after 48 h of treatment. DAPI stains for nuclei. The microscopes show representative fields of each treatment for three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

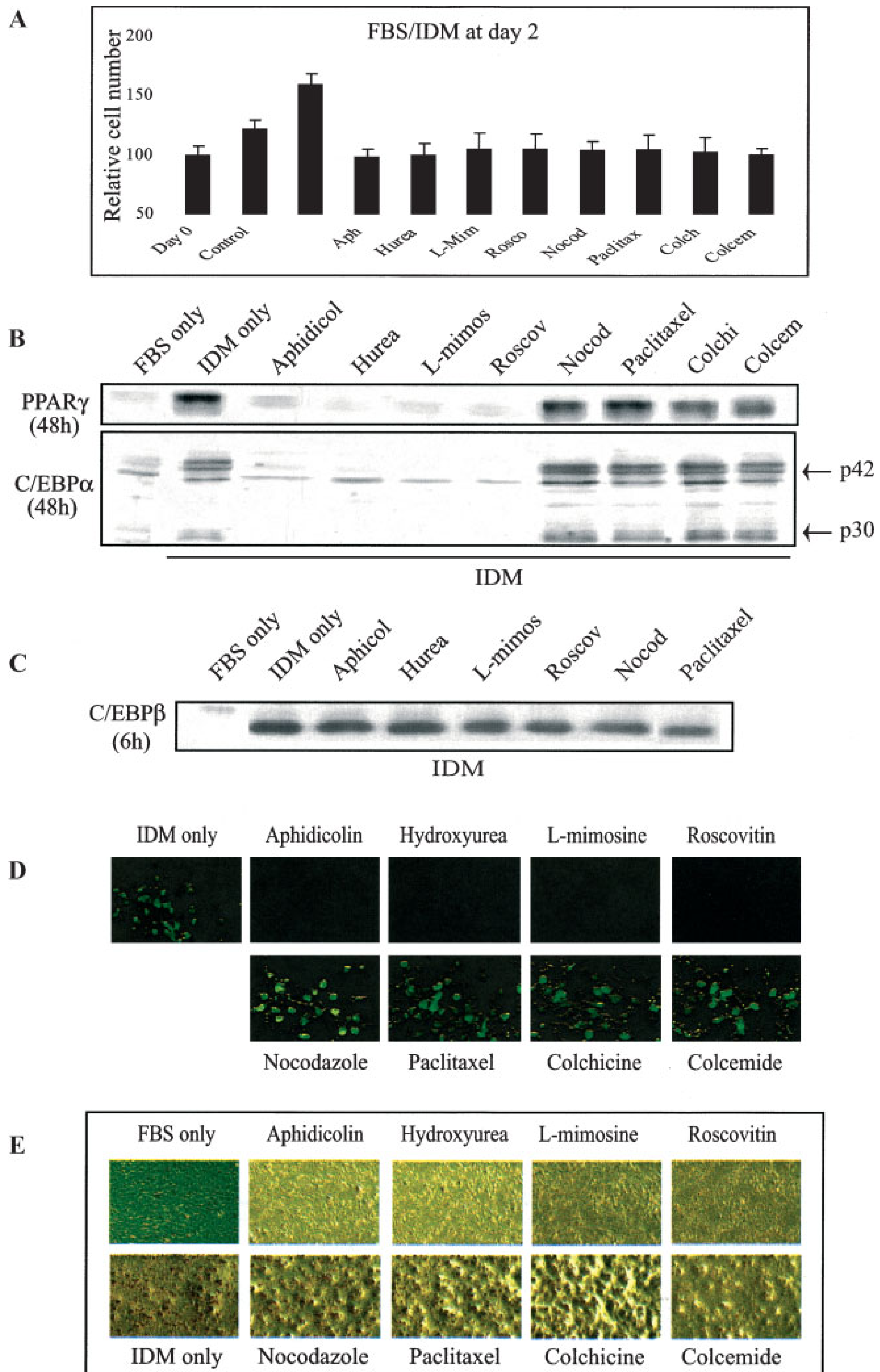

Fig. 5.

Inhibition of Cell Cycle Progression Through S-Phase Prevents PPARγ Expression and Lipogenesis

To test the possibility that differentiation was linked to cell cycle progression, we used cell cycle inhibitors, which selectively caused cell cycle arrest at the S- or G2/M-phase, respectively. The S-phase inhibitors each act by different mechanisms: DNA polymerase inhibition (aphidicolin), ribonucleotide reductase inhibition (hydroxyurea), DNA intercalation (L-mimosine), and cyclin dependent kinase inhibition (roscovitin) [Cheng and Kuchta, 1993; De Azevad et al., 1997; Hendericks and Mathews, 1998; Krude, 1999]. The G2/M inhibitors (nocodazole, paclitaxel, colchicine, and colcemide) each arrest cell cycle and block mitosis by inhibiting microtubule assembly [Luduena and Roach, 1991; Nishiyama and Fujii, 1992; Parekh and Simpkin, 1997; DeVincenzo et al., 1998]. Each inhibitor completely blocked mitotic clonal expansion stimulated by the standard IDM mixture (Fig. 5A). Expression of PPARγ and C/EBPα stimulated by IDM (48 h) was completely prevented by each the four S-phase inhibitors (Fig. 5B), while G2/M-phase inhibitors had no effect on either PPARγ or C/EBPα expression (Fig. 5B). In contrast, S- and G2/M-phase inhibition did not affect the optimal induction of C/EBPβ observed 6 h after IDM stimulation (Fig. 5C). Exactly the same discrimination between S- and G2/M-phase inhibition was found for lipogenesis, as measured by Oil-Red-O staining (Fig. 5D). The same effects of these inhibitors on PPARγ, C/EBPα, and lipogenesis were obtained using DM stimulation or when the mitogenic stimulation from fresh serum was omitted (URS protocol, data not shown). These results establish that mitotic clonal expansion is not an essential prerequisite for either PPARγ expression or adipocyte differentiation. By contrast, prevention of DNA replication by S-phase arrest indicates that DNA synthesis is essential for adipogenic induction.

DM Treatment Causes an Apparent G2/M-Phase Accumulation, but no Mitosis

BrdU incorporation, stimulated by the standard IDM/FBS mixture (12–15 h period post treatment), was unaffected by G2/M-phase inhibitors, but was completely prevented by S-phase inhibitors (Fig. 5E). Heterogeneity in DNA labeling was particularly evident in the experiment with the G2/M-phase inhibitors. To further define changes in the cell cycle distribution regulated by these treatments. FACS analyses were carried out 16 h after hormonal stimulation, the period immediately following peak DNA synthesis (Fig. 6). Under most conditions, 70–80% of cells were in G0/G1-phase. IDM/FBS stimulation lowered the proportion of cells in S-phase (from 18 to 3%), while increasing cell cycle progress to G2/M-phase (from 4 to 19%). A proportion of these cells ultimately divided and, therefore, completed mitosis. Omission of insulin, which had little effect on DNA synthesis (Fig. 4C), also had very little effect on this cell cycle re-distribution, in spite of the loss of cell division. Omission of serum renewal (DM/URS) produced a comparable redistribution from S-phase to G2/M-phase, in spite of lower DNA synthesis. Thus, the effects of insulin and serum on DNA synthesis and mitosis have little impact on the proportion of cells directed to the G2/M-phase by the combination of DEX and MIX, except for a modest decrease under mitotic conditions (FBS/IDM versus URS/DM). The G2/M inhibitors produced a

Fig. 5. (*Overleaf*) DNA synthesis, but not mitotic clonal expansion is an essential step for adipogenesis. **A**: Inhibition of mitosis. Post-confluent cells were treated for 48 h with the IDM mixture in medium supplemented with fresh FBS, in the presence of G1/S inhibitors (10 μM aphidicolin (Aph), 3 mM hydroxyurea (Hurea), 100 μM L-mimosine (L-mim), or 25 μM roscovitin (Rosco)) or G2/M inhibitors (2.5 μg/ml nocodazole (Nocod), 1 μM paclitaxel (Paclitax), 100 nM colchicines (Colch), or 1 μM colcemide (Colcem)). Cell numbers were determined 2 days following hormonal stimulation. Experiments were done in triplicate with two different times. The average cell number at day zero (day 0) is normalized to 100. Data are presented as average ± SD. **B**: Expression of PPARγ and C/EBPα after 48 h. Treated cells were harvested and total cell lysate (50 μg) was subjected to Western blot analysis with polyclonal antibody for C/EBPα or monoclonal PPARγ antibody. The Western blot shown is representative of three independent experiments. **C**: Expression of CCAAT/enhancer binding protein β (C/EBPβ) after 6 h. Western blot analysis with polyclonal C/EBPβ antibody. **D**: Immunofluorescence analysis of BrdU incorporation. BrdU was pulsed from the 12–15 h after hormonal treatment. BrdU labeling was visualized after 48 h. DAPI stains for nuclei. The microscopes show representative fields of each treatment for three independent experiments. **E**: Lipogenesis after day 8. Oil-Red-O staining for cytoplasmic triglyceride droplets. Post-confluent cells were incubated with the IDM mixture in the presence of G1/S and G2/M inhibitors, as described above. The microscopes show representative fields of each treatment for three or more independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

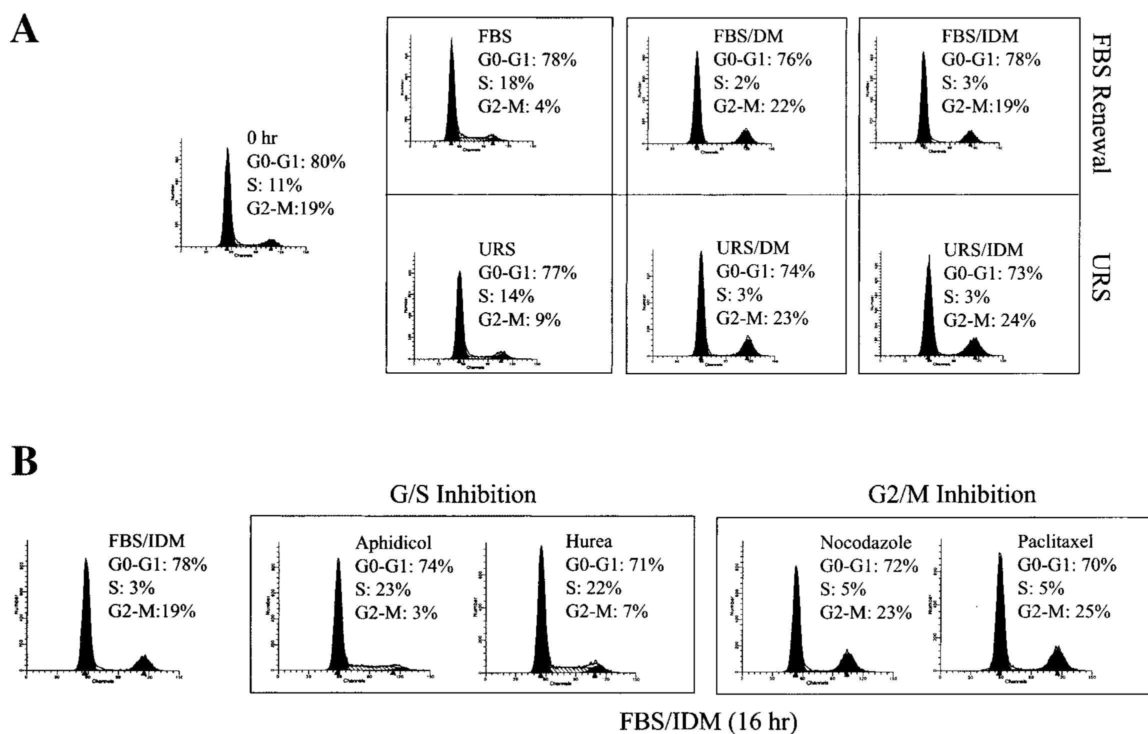

Fig. 6. S-phase block prevents adipogenesis independently of mitotic clonal expansion. **A**: Effects of hormones and serum renewal. Cells were treated with the hormonal mixture in medium containing fresh serum (FBS) or URS, as described in Figure 1C. After 16 h, treated cells were harvested and incubated with propidium iodide (33 ng/ml) for fluorescence activated cell sorting (FACS) cell cycle analysis. Experiments were done in duplicate with two different times. Data are presented as average. **B**: Effects of cell cycle inhibition. Cells were treated with IDM/FBS in the presence of G1/S inhibitors (aphidicolin and hydroxyurea) or G2/M inhibitors (nocodazole and paclitaxel) and were analyzed after 16 h. Experiments were done in duplicate with three different times. Data are presented as average.

remarkably similar proportion of cells in G2/M consistent with a comparable inhibition by DM. As expected, the S-phase inhibitors prevented the increase in G2/M-phase, while producing a comparable increase in S-phase. Similarly, cell cycle accumulation in either S-phase or G2/M-phase was also seen 24 h after hormonal stimulation of each type (data not shown). By contrast, proportion of S-phase increased while G2/M-phase accumulation was attenuated 24 h after IDM/FBS stimulation indicating passage through mitosis (data not shown).

Based on the diversity of DNA synthesis measured immediately prior to the FACS assessment, these cell cycle re-distribution analyses represent the average of heterogeneous responses, including up to half of the cells remaining unresponsive in G0/G1-phase.

Cell Confluence Is not an Essential Condition for Adipocyte Differentiation

The heterogeneity of adipocyte differentiation of 10T1/2 cells, including the clonally isolated sub-lines, raised questions of whether cell–cell contacts provide a unique environment for each individual cell. We, therefore, tested whether LD cultures, in which most cells are fully separated, respond to adipogenic stimulation. Prior to addition of the IDM mixture, cells were dispersed at about 10% of confluence for 2 days. In LD culture, approximately 50% of the cells were in S-phase, consistent with active proliferation (data not shown). The sub-confluent culture consisted of single-isolated cells and clusters of two to three cells. IDM stimulation was carried out in the medium with fresh serum renewal (LD/FBS) or without serum renewal (LD/URS) and was continued for upto 96 h (day 4) without medium change (Fig. 7A). Induction of PPARγ expression and lipogenesis in these LD cells was effective under both stimulation conditions and, indeed, was equivalent to that seen in confluent cells tested with the standard protocol (IDM for first 2 days and following 2 days with insulin replacement) (Fig. 7A,C).

Cell proliferation was distinctively observed in a set of separate LD colonies, which, however,

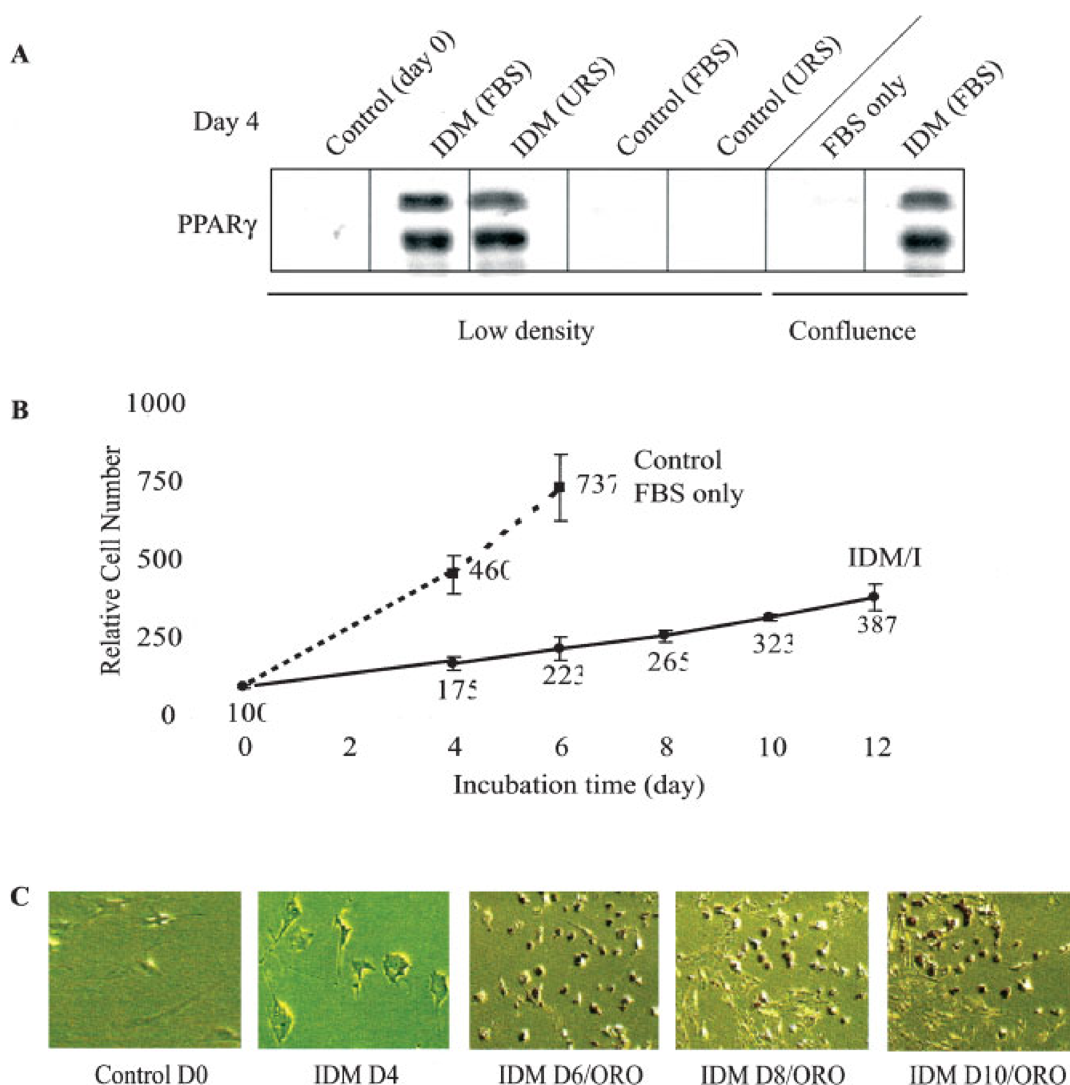

Fig. 7. Cell confluence is not an essential condition for adipocyte differentiation, in vitro; actively growing cells are committed to adipocyte differentiation in sub-confluent culture. **A**: PPARγ expression. Cells were cultured for 2 days to approximately 10% confluence and treated with IDM in medium supplemented with fresh FBS (IDM/FBS) or without serum renewal (IDM/URS) for 96 h without medium change. At day 4, treated cells were lysed and harvested. The same batch of cells was similarly treated at confluence. Lysates were subjected to Western blot analysis with monoclonal antibodies for PPARγ. The Western blot shown is representative of three independent experiments. **B**: Mitosis. Cell numbers were determined on the indicated days. Experiments were done in triplicate. The average cell number at day zero (day 0) is normalized to 100. Data are presented as average ± SD. **C**: Lipogenesis. Phase contrast images of sub-confluent 10T1/2 cells measure adipogenic induction using Oil-Red-O stain on the indicated days. Sub-confluent cells were incubated with IDM/FBS mixture for 96 h, and then the medium was renewed with insulin and fresh FBS every 2 days. The microscopes show representative fields of each treatment for three or more independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

did not show any lipogenesis. In the absence of IDM stimulation, LD cells increased more than seven-fold and reached near confluence in a 6 day period, with or without serum renewal (Fig. 7B and data not shown). A limited cell proliferation was shown during the first 4 days under IDM stimulation that was, however, progressing at a far slower rate than in FBS medium (Fig. 7B). When the IDM mixture was replaced, after day 4, with insulin and serum renewal, lipid droplets appeared by day 6 in approximately half of the cells (Fig. 7C). Importantly, differentiating cells did not proliferate, while many non-differentiating cells resumed cell proliferation (Fig. 7B,C). There was no indication of lipid droplets in the expanding

colonies. Mitotic clonal expansion and differentiation in 10T1/2 cells, therefore, function as completely distinct processes.

Interestingly, DM stimulation suppressed cell growth in LD cells much like IDM, but, in contrast to confluent cells, induced very little adipogenesis (data not shown). Evidently, there is a greater need for insulin at adipogenic induction in this LD culture.

We further examined whether DNA synthesis or clonal expansion in LD cells was required for adipocyte differentiation with IDM stimulation. Both S- and G2/M-phase inhibition completely blocked mitotic clonal expansion (Fig. 8A). While PPARγ expression and lipogenesis were not achieved in the presence of S-phase inhibitors, G2/M-phase inhibition permitted full expression of PPARγ and adipocyte differentiation (Fig. 8B,C). These results indicate that S-phase progression remains essential for adipcyte differentiation in sub-confluent 10T1/2 cells, while mitotic clonal expansion is unnecessary. Figure 8D summarizes the requirements for culture conditions and responses to adipogenic stimulation in confluent and LD cells.

DISCUSSION

In this study, we establish that mitotic clonal expansion is not necessary for adipocyte differentiation of 10T1/2 cells, whereas maintenance of DNA synthesis and cell cycle progression through S-phase in the presence of hormonal stimuli are essential. The 10T1/2 cells undergo maximum adipogenesis after removal of the major mitogenic signals, provided by insulin and fresh serum supplementation, from the standard IDM protocol (Figs. 1 and 3). Hormonal treatment without mitogenic stimuli (DM without serum renewal; DM/URS), indeed, prevented mitotic clonal expansion, while maintaining maximum stimulation of key adipogenic regulators, including the PPARγ1 isoforms, C/EBPα, and activated ADD1/SREBP1, and subsequent insulin-dependent lipogenesis. The early influence of insulin on these regulatory factors was limited to a stimulation of PPARγ2 expression followed by an activation of PPARγ1, which could even be replaced by the PPARγ ligand, BRL. We have also used selective cell cycle inhibitors to further establish that entry into S-phase is essential for PPARγ synthesis and lipogenesis, but that passage through mitosis is unnecessary. Indeed, optimal differentiation by DM stimulation alone is accompanied by a similar accumulation of cells in G2/M-phase.

Although insulin-induced stimulation of DNA synthesis was unimportant to the elevation of PPARγ1 and C/EBPα expression, DNA synthesis was not only detectable without insulin, but essential (Fig. 4). Increases of DNA synthesis, however, do not parallel mitogenesis, PPARγ1 expression, or differentiation. While insulin functioned synergistically with serum renewal to maximally stimulate a 3 h burst of DNA synthesis after 12 h (Fig. 4), a more limited burst of DNA synthesis was produced by DM alone without serum renewal (DM/URS). We confirmed the functional linkage between PPARγ expression and lipogenesis by showing that each occurred exclusively in the same cells (Fig. 2). This maximum stimulation of PPARγ1 and lipogensis was, nevertheless, completely blocked by each of four mechanistically distinct DNA synthesis inhibitors. We conclude that only low levels of DNA synthesis are necessary, but, under conditions of confluent arrest, this may require stimulation by DM. This, however, is only sufficient in the half of the cells that engage in differentiation.

We have completely dissociated PPARγ induction and lipogenesis from mitotic clonal expansion by means of G2/M-specific inhibitors (Fig. 5). Each completely inhibits mitotic clonal expansion, while fully maintaining elevated PPARγ and C/EBPα expressions and adipocyte conversion. These inhibitors did not affect the DNA synthesis, although an enhanced proportion of the stimulated cells got arrested, as expected, in the G2/M-phase (Fig. 6). S-phase specific inhibitors completely block PPARγ expression and adipogenesis without affecting C/EBPβ induction by IDM, which appears after approximately 6 h of stimulation (Fig. 5). The addition of the DNA polymerase inhibitor, aphidicolin, to IDM-stimulated cells after this rise in C/EBPβ remained effective in preventing PPARγ induction and adipogenesis (data not shown). Evidently, the aphidicolin-sensitive step occurs after C/EBPβ induction, which could still include the period of the elevated DNA synthesis. Interestingly, previous study on 3T3-L1 cells clearly shows that C/EBPβ induction, as measured by DNA binding, is a later event that would be expected to overlap with the period of elevated DNA synthesis observed in this study [Tang et al., 2003].

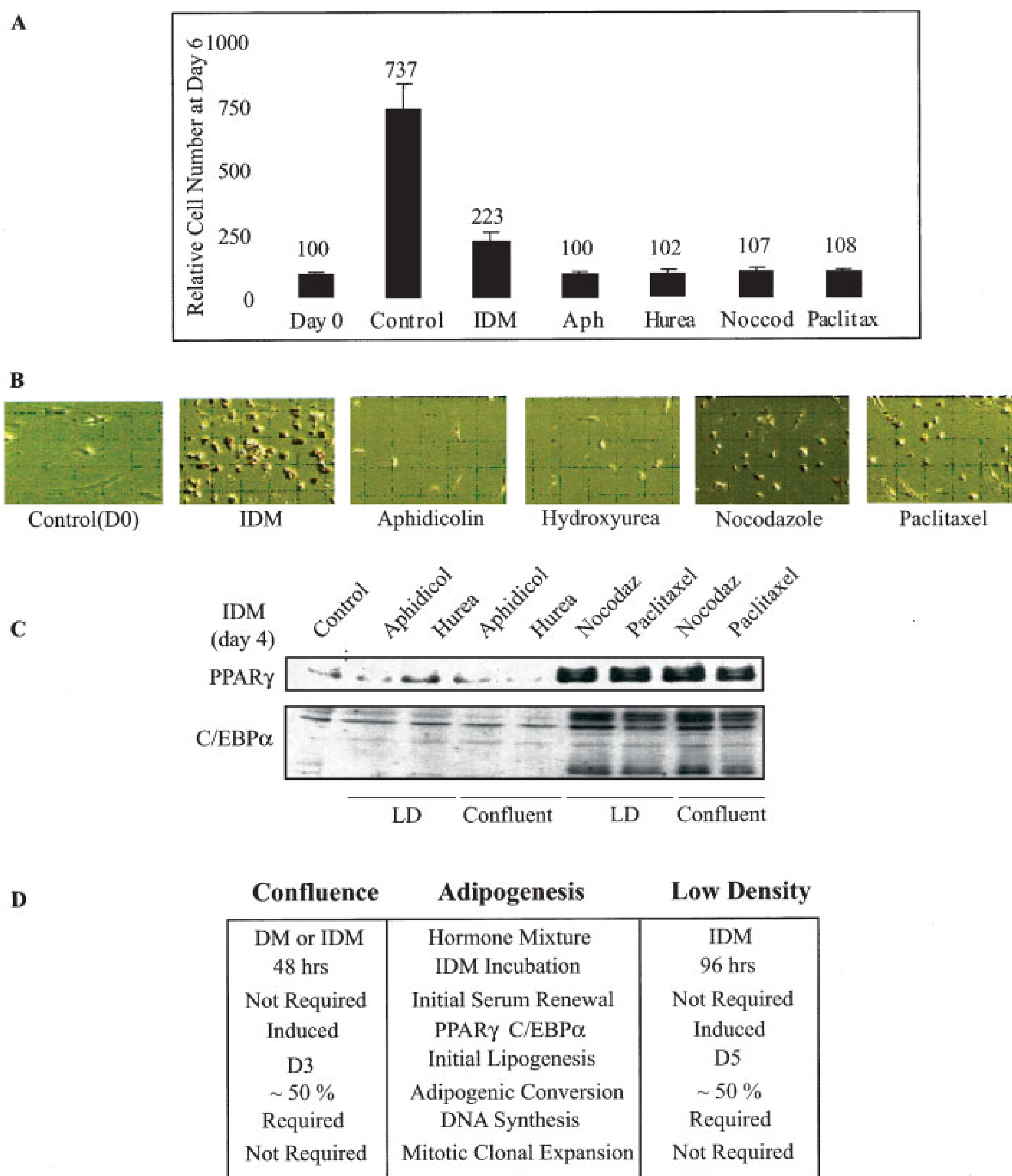

Fig. 8. DNA synthesis, but not mitosis, is essential for adipogenesis in sub-confluent cells. **A**: Inhibition of mitosis by cell cycle inhibition. Sub-confluent 10T1/2 cells were incubated with the IDM/FBS or IDM/URS mixture in the presence of G1/S inhibitors (aphidicolin or hydroxyurea) or G2/M inhibitors (nocodazole or paclitaxel) for 96 h, as described in Figure 7A. Cell numbers were counted at either day 0 or day 6. Experiments were done in triplicate. The average cell number at day zero (day 0) is normalized to 100. Data are presented as average ± SD. **B**: Lipogenesis without mitotic clonal expansion. Phase contrast images of sub-confluent 10T1/2 cells stained with Oil-Red-O at day 8 for the measurement of adipogenic induction. The microscopes show representative fields of each treatment for three or more independent experiments. **C**: Expression of PPARγ and C/EBPα. Total cell lysate (50 μg) from sub-confluent or confluent culture, harvested at day 4 as described in Figure 5B or 7A, was subjected to Western blot analysis with polyclonal antibody for C/EBPα or monoclonal PPARγ antibody. The Western blot shown is representative of three independent experiments. **D**: Summary. Requirements of culture conditions and responses to adipogenic stimulation in confluent and low density (LD) cultures. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

S-phase inhibitors activate distinct signal pathways mediated by ATM/ATR, such as Chk protein phosphorylation, which are responsive to impaired or stalled DNA synthesis [Abraham, 2001]. Such adaptive responses caused by the dysfunctional DNA synthesis may also inhibit the activation of PPARγ transcription. Thus, aphidicolin effects the activation of ATM/ATR kinases, which initiate a cascade through Chk1 and Chk2 proteins, blocking the cell cycle progression directed by cyclin B/Cdk2 and Cdc25 [Abraham, 2001]. Currently, we can't distinguish this type of adaptive inhibitory mechanism from an absolute requirement of DNA synthesis in adipocyte differentiation.

The contribution of DNA synthesis to the DM stimulation of PPARγ1 expression and cell cycle exit is complex. There are substantial differences in the extent of BrdU incorporation that accompany similar levels of adipogenesis. The 12–15 h period of DNA synthesis ranges from IDM/FBS stimulation where approximately half of the cells respond to DM/URS, where only a few cells respond weakly (Fig. 4C). The diversity of these responses, while demonstrating equivalent levels of differentiation, confirms that a full DNA synthesis response is not a step along the differentiation pathway. We have also shown that each adipogenic stimulus effects a similar cell cycle accumulation in G2/M-phase, which is almost comparable to the effect of the G2/M inhibitors. We speculate that there is a checkpoint at some time during S-phase where the stimulated cells may be segregated between commitment to a differentiation-specific pathway and continuation to mitosis. Interestingly, at day 4, after 2 days of insulin-induced lipogenesis, about half of the cell population showed DNA aneuploidy, while control cells mostly were in G0/G1-phase (data not shown).

Adipogenic induction of 10T1/2 cells follows a remarkably similar time course for generation of the various regulatory factors when compared to the more rapidly responding 3T3-L1 cells [Hamm et al., 2001]. By contrast, other types of 3T3-L1 cells exhibit a substantially delayed production of PPARγ [Tang et al., 2003]. The proportion of cells converted to adipocytes is, however, substantially lower with the 10T1/2 cell line. The basis for this difference between 3T3-L1 and 10T1/2 cells is not well understood. This partial conversion is retained in multiple sub-lines of 10T1/2 cells, which are clonally isolated and, thus, are genetically identical (Fig. 2A). Adipogenic conversion occurs in approximately 50% of the cells cultured from these clonal sub-lines, which seems to represent an intrinsic partitioning of the pluripotent 10T1/2 lineage between mitotic and differentiation pathways.

Surprisingly, a similar pattern of differentiation was retained in sub-confluent cultures of 10T1/2 cells (LD), in which cell–cell contacts were absent (Fig. 7). This has allowed us to examine the division between differentiation and mitotic clonal expansion, independent of the constraints imposed in confluent cultures. Under these LD conditions, IDM treatment effectively induced PPARγ1 and caused a commitment to insulin-dependent lipogenesis, which paralleled the response observed in confluent cells. Evidently, cell–cell contacts and preliminary cell cycle exit by contact inhibition, which characterizes confluent cells, are not necessary for adipocyte differentiation in 10T1/2 cells, even though they may contribute to the high density process. The IDM cocktail again segregates cells into roughly equal groups that either arrest and undergo differentiation or exhibit a progressive clonal expansion. No adipocytes appeared in these expanding colonies. We will, however, describe elsewhere that these cells retain the full differentiation response to the IDM stimulation. G2/M-phase inhibition during IDM stimulation again had no effect on the PPARγ and C/EBPα expression levels or on lipogenesis in isolated cells, but prevented the expansion of the non-differentiating colonies. Again, S-phase inhibition completely prevented both adipogenesis and the clonal expansion. This replication of differentiation characteristics at LD indicates that adipogenic commitment is not determined by signaling associated with cell–cell contacts. However, unlike the stimulation of confluent cells, the LD processes exhibited an absolute requirement for insulin in the initial 2 days.

In conclusion, we propose that S-phase progression mediated by hormonal stimulation is an essential step for the activation of PPARγ1 synthesis, which seems to limit cell cycle exit and support the commitment to lipogenesis. DNA synthesis appears to be necessary for this step, although possibly only through a low level activity that is monitored by the cells to allow entry into S-phase. During or after S-phase progression, hormonally stimulated cells become destined either to mitotic clonal expansion or to

PPARγ1 synthesis and adipogenic commitment. Each of the conditions that stimulate this differentiation pathway causes substantial G2/M-phase accumulation. Progression to this stage of the cell cycle may be necessary or possibly this same G2/M accumulation signal may mediate commitment to the differentiation pathway at an earlier stage. The retention of very similar processes in isolated, sub-confluent cells emphasizes that that this programming is autonomous to each individual cell.

The recent publications on 3T3L1 cells [Qiu et al., 2001; Tang et al., 2003] are not inconsistent with these findings. The 3T3-L1 cells, which express PPARγ1, rapidly (during initial 48 h) differentiate mitosis [Qiu et al., 2001], while the variant 3T3-L1 sub-line, which is reported to require mitosis [Tang et al., 2003] exhibits a delayed rise in PPARγ synthesis, possibly because one or more rounds of mitosis are necessary. In these slow responding 3T3-L1 cells, mitosis is probably necessary to release epigenetic constraints on the transcriptional activation of PPARγ1, which appears to be delayed. The critical issue in the context of the mechanism proposed here is whether, in the cell cycle progression after mitotic expansion, diversion to PPARγ1 synthesis starts in S-phase. The common feature in each case seems to be the need for cell cycle progression, which, as previously proposed, may play an essential role in C/EBPβ activation [Hamm et al., 2001].

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REFERENCES

Abraham RT. 2001. Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev 15:2177–2196.

Alexander DL, Ganem LG, Fernandez-Salguero P, Gonzalez F, Jefcoate CR. 1998. Arylhydrocarbon receptor is an inhibitory regulator of lipid synthesis and of commitment to adipogenesis. J Cell Sci 111:3311–3322.

Altiok SM, Xu M, Spiegelman BM. 1997. PPARγ induces cell cycle withdrawal: Inhibition of E2F/DP DNA-binding activity via downregulation of PP2A. Genes Dev 11: 1987–1998.

Cao Z, Umek RM, McKnight SL. 1991. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. Genes Dev 5:1538–1552.

Chawla A, Lazar MA. 1994. Peroxisome proliferator and retinoid signaling pathways co-regulate preadipocyte phenotype and survival. Proc Natl Acad Sci USA 91: 1786–1790.

Cheng CH, Kuchta RD. 1993. DNA polymerase epsilon: Aphidicolin inhibition and the relationship between polymerase and exonuclease activity. Biochemistry 32:8568–8574.

Christy RJ, Yang VW, Ntambi JM, Geiman DE, Landschulz WH, Friedman D, Lane MD. 1989. Differentiation-induced gene expression in3T3-L1 preadipocytes: CCAAT/enhancer binding protein interacts with and activates the promoters of two adipocyte-specific genes. Genes Dev 3:1323–1335.

De Azevad WF, Leclerc S, Meijer L, Havlicek L, Strnad M, Kim SH. 1997. Inhibition of cell cycle-dependent kinase by purine analogues: Crystal structure of human cdk2 complexed with roscovitin. Eur J Biochem 243:518–526.

DeVincenzo R, Scambia G, Ferlini C, Distefano M, Filippini P, Riva A, Bombardelli E, Pocar D, Gelmi ML, Benedetti-Panici P, Mancuso S. 1998. Antiproliferative activity of colchicines analogues on MDR-positive and MDR-negative human cancer cell lines. Anticancer Drug Des 13:19–33.

DiRenzo J, Soderstrom M, Kurokawa R, Ogliastro MH, Ricote M, Ingrey S, Horlein A, Rosenfeld MG, Glass CK. 1997. Peroxisome proliferator-activated receptors and retinoic acid receptors differentially control the interactions of retinoid X receptor heterodimers with ligands, coactivators and corepressors. Mol Cell Biol 17:2166–2176.

Font de Mora J, Porras A, Ahn N, Santos E. 1997. Mitogen-activated protein kinase activation is not necessary for, but antagonizes, 3T3-L1 adipocyte differentiation. Mol Cell Biol 17:6068–6075.

Freytag SO, Paielli DL, Gilbert JD. 1994. Ectopic expression of the CCAAT/enhancer-binding protein α promotes the adipogenic program in a variety of mouse fibroblastic cells. Genes Dev 8:1654–1663.

Green H, Kehinde O. 1975. An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. Cell 5:19–27.

Green H, Meuth M. 1974. An established pre-adipose cell line and its differentiation in culture. Cell 3:127–133.

Gregoire FM, Smas CM, Sul HS. 1998. Understanding adipocyte differentiation. Physiol Rev 78:783–809.

Hamm JK, Park BH, Farmer SR. 2001. A role for C/EBPβ in regulating peroxisome proliferator-activated receptor γ activity during adipogenesis in 3T3-L1 areadipocytes. J Biol Chem 276:18464–18471.

Hendericks SP, Mathews CK. 1998. Differential effects of hydroxyurea upon deoxyribonucleoside triphosphate pools analyzed with vaccinia virus ribonucleotide reductase. J Biol Chem 273:29519–29523.

Kim JB, Spiegelman BM. 1996. ADD1/SREBP1 promotes adipocyte differentiation and gene expression linked to fatty acid metabolism. Genes Dev 10:1096–1107.

Kim JB, Wright HM, Wright M, Spiegelman BM. 1998. ADD1/SREBP1 activates PPARγ through the production of endogenous ligand. Proc Natl Acad Sci USA 95:4333–4337.

Kim SW, Muise AA, Lyons PJ, Ro HS. 2001. Regulation of adipogenesis by a transcriptional repressor that modulates MAPK activation. J Biol Chem 276:10199–10206.

Konieczny SF, Emerson CP, Jr. 1984. 5-Azacytidine induction of stable mesodermal stem cell lineages from 10T1/2 cells: Evidence for regulatory genes controlling determination. Cell 38:791–800.

Krude T. 1999. Mimosine arrest proliferating human cells before onset of DNA replication in a dose-dependent manner. Exp Cell Res 247:148–159.

Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Wilson TM, Kliewer SA. 1995. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferation-activated receptor γ (PPARγ). J Biol Chem 270:12953–12956.

Luduena RF, Roach MC. 1991. Tubulin sulfhydryl groups as probes and targets for antimitotic and antimicrotubule agents. Pharmacol Ther 49:133–152.

Morrison RF, Farmer SR. 1999. Role of PPARγ in regulating a cascade expression of cyclin-dependent kinase inhibitors, p18 (INK4C), and p21 (Waf-1/Cip-1), during adipogenesis. J Biol Chem 274:17088–17097.

Nishiyama I, Fujii T. 1992. Laminin-induced process out growth from isolated fetal rat C-cells. Exp Cell Res 198:214–220.

Parekh H, Simpkin H. 1997. The transport and binding of taxol. Gen Pharmacol 29:167–172.

Párrizas M, Saltiel AR, LeRoith D. 1997. Insulin-like growth factor inhibits apoptosis using the phosphatidylinositol 3′-kinase and mitogen-activated protein kinase pathways. J Biol Chem 272:154–161.

Qiu Z, Wei Y, Chen N, Jiang M, Wu J, Liao K. 2001. DNA synthesis and mitotic clonal expansion is not required step for 3T3-L1 preadipocyte differentiation into adipocytes. J Biol Chem 276:11988–11995.

Schluman IG, Shao G, Heyman RA. 1998. Transactivation by retinoid X receptor-peroxisime proliferator-activated receptor gamma (PPARγ) heterodimers: Intermolecular synergy requires only the PPAR gamma hormone-dependent activation function. Mol Cell Biol 18:3483–3494.

Shao D, Lazar MA. 1997. Peroxisome proliferator activated receptor gamma, CCAAT/enhancer-binding protein alpha, and cell cycle status regulate the commitment to adipocyte differentiation. J Biol Chem 272:21473–21478.

Tang QQ, Otto TC, Lane MD. 2003. Mitotic clonal expansion: A synchronous process required for adipogenesis. Proc Natl Acad Sci USA 100:44–49.

Taylor SM, Jones PA. 1979. Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. Cell 17:771–779.

Timchenko NA, Wilde M, Nakanish M, Smith JR, Darlington GJ. 1996. CCAAT/enhancer-binding protein alpha (C/EBP alpha) inhibits cell proliferation through p21 (WAF-1/CIP-1/SDI-1) protein. Genes Dev 10:804–815.

Tontonoz P, Hu E, Spiegelman BM. 1994a. Stimulation of adipogenesis in fibroblasts by PPARγ2, a lipid-activated transcription factor. Cell 79:1147–1156.

Tontonoz P, Hu E, Graves RA, Budavari AI, Spiegelman BM. 1994b. mPPARγ2: Tissue-specific regulator of an adipocyte enhancer. Genes Dev 8:1224–1234.

Umek RM, Friedman AD, McKnight SL. 1991. CCAAT-enhancer binding protein: A component of a differentiation switch. Science 251:288–292.

Whithers DJ, White M. 2000. Insulin signaling system—A common link in the pathogenesis of type 2 diabetes. Endocrinology 141:1917–1921.

Wu Z, Xie Y, Bucher NLR, Farmer SR. 1995. Conditional ectopic expression of C/EBP beta in NIH-3T3 cells induces PPAR gamma and stimulates adipogenesis. Genes Dev 9:2350–2363.

Wu Z, Rosen ED, Brun R, Hauser S, Adelmant G, Troy AE, McKeon C, Darlington GJ, Spiegelman BM. 1999. Cross regulation of C/EBPα and PPARγ controls the transcriptional pathway of adipogenesis and insulin sensitivity. Mol Cell 3:151–158.