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Coupling of growth arrest and differentiation at a distinct state in the G_1 phase of the cell cycle: G_D

(proadipocytes/growth control/carcinogenesis)

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ABSTRACT **The differentiation of most mammalian cells is preceded by growth arrest in the G_1 phase of the cell cycle, but the characteristics of this state have not been established. We now report that the growth arrest that precedes the differentiation of BALB/c 3T3 T mouse proadipocytes must occur at a distinct state in G_1 designated G_D. G_D-arrested cells are characterized by their ability to differentiate in the absence of DNA synthesis and by their unique sensitivity to the mitogenic effect of isobutylmethylxanthine. Proadipocytes induced to become G_1 growth arrested at other states by culture in medium deficient in growth factor or nutrients, by contrast, are unable to differentiate in the absence of DNA synthesis and are not stimulated to proliferate by isobutylmethylxanthine even when they are exposed to differentiation-promoting medium prior to arrest. These data support the conclusion that, prior to the expression of a differentiated phenotype, proadipocytes must arrest their growth at a distinct state in the G_1 phase of the cell cycle, G_D. These data also provide the basis for the hypothesis that carcinogenesis is associated with defects in the coupling of growth arrest and differentiation at the G_D state.**

Regulation of cell proliferation of many cell types is mediated by the coupling of growth arrest and differentiation. *In vivo* studies on hematopoietic cells (1) and epithelial cells of the skin (2) and *in vitro* studies on other cell types (3) have established this fact. Although there is considerable controversy concerning the mechanisms that serve to initiate the differentiation process (4), the available evidence suggest growth arrest in the G_1 phase of the cell cycle precedes expression of the differentiated phenotype. It has, however, not been established whether growth arrest occurs at a distinct state in G_1 prior to differentiation. This question is of critical importance if the metabolic events that control the coupling of growth arrest and differentiation are to be established and if defects in the coupling process that may be associated with various disease states, such as aging and carcinogenesis, are to be identified. In this paper we report on the first of our studies, which show that, prior to differentiation, proadipocytes arrest their growth at a state in the G_1 phase of the cell cycle (G_D) that is distinct from the G_1 growth arrest states induced by deprivation of growth factor or serum (G_S) or by deprivation of nutrients (G_N).

MATERIALS AND METHODS

Cell Culture, Cell Proliferation, and Cell Differentiation. The proadipocyte cell line, designated 3T3 T, derived from BALB/c 3T3 (A31) mouse embryo cells by L. Diamond (5), was grown at 37°C in a 5% CO_2 humidified atmosphere in Dulbecco's modified Eagle's medium (DME medium) containing 10% heat inactivated fetal calf serum unless otherwise stated. All stock cultures were grown in antibiotic-free medium; experimental specimens, however, were cultured in medium supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were periodically shown to be free of mycoplasma contamination (6). Cell density analyses were performed with a Coulter Counter or by microscopic assays. Incorporation of [^{3}H]thymidine into DNA was used as an assay for cells in the S phase of the cell cycle. [^{3}H]Thymidine incorporation was determined by scintillation or autoradiographic methods (7). Adipocyte differentiation was quantitated by morphological and enzymatic assays. For morphological assays cells were fixed, stained with oil red O, and counterstained with 0.1% toluidine blue. The percent fat cells and the number of fat cells per cm^2 were determined by random counts of 300–500 cells. Enzyme assays for both lipoprotein lipase and glycerol-3-phosphate dehydrogenase activities were performed as described (8, 9). Both enzymes have been shown to be excellent markers for adipocyte differentiation (10). Protein assays were performed by the method of Lowry *et al.* (11).

Preparation of G_D-, G_S-, and G_N-Arrested Cells. Proadipocytes were G_D arrested by two methods.

High-density G_D. This method produced G_D-arrested cells at a density of $\approx 1 \times 10^5$ cells per cm^2. Cells were grown in 100% confluence in DME medium containing 10% fetal calf serum. Cells were then cultured in DME medium containing 30% fetal calf serum and insulin at 50 μg/ml for 6–8 days.

Low-density G_D. This method produced G_D-arrested cells at a density of $\approx 2 \times 10^4$ cells per cm^2. Cells were grown to 10–20% confluence in DME medium containing 10% fetal calf serum. These rapidly growing cells were then cultured in heparinized DME medium containing 25% human plasma for 4–8 days.

G_S-arrested cells were prepared at three different densities.

Low-density G_S. This method produced G_S-arrested cells at a density of $\approx 2 \times 10^4$ cells per cm^2. Cells were grown to 10–15% confluence in DME medium/10% fetal calf serum. The medium was decanted and the cells were rinsed with isotonic phosphate-buffered saline and then cultured in DME medium/0.5% fetal calf serum for 4–8 days.

Confluent-density G_S. This method produced G_S-arrested cells at a density of $\approx 5 \times 10^4$ cells per cm^2. Cells were grown to confluence in DME medium containing 10% fetal calf serum and maintained therein until spontaneous growth arrest occurred.

High-density G_S. Two methods were used to produce proadipocytes G_S arrested at a density of $\approx 1 \times 10^5$ cells per cm^2.

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Abbreviations: DME medium, Dulbecco's modified Eagle's medium; PPP-S, platelet-poor-plasma-derived serum; HHP, heparinized medium containing human plasma; BaP, barium-citrate-treated plasma; CEP, citrate eluate of plasma components that adsorbed to barium citrate precipitate; EDTAP, EDTA eluate of plasma components that adsorbed to barium citrate precipitate; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$.

First, 70% confluent rapidly growing cells were trypsinized and replated in DME medium/10% fetal calf serum at a density of $\approx 1 \times 10^5$ cells per cm^2. These cells attached to the substratum, formed a high-density monolayer, and arrested their growth. Second, confluent cells grown in DME medium/10% fetal calf serum were exposed to epidermal growth factor at 1–5 ng/ml at daily intervals until a density of $\approx 1 \times 10^5$ cells per cm^2 was achieved.

G_N-arrested cells were prepared at two densities by culture in isoleucine-deficient DME medium supplemented with 10% fetal calf serum that had been extensively dialyzed to remove nutrients and other factors with molecular weights less than approximately 10,000 (12).

Low-density G_N. This method produced G_N-arrested proadipocytes at a density of $\approx 2 \times 10^4$ cells per cm^2. Cells were grown to ≈20% confluence in DME medium/10% fetal calf serum. They were then cultured in isoleucine-deficient DME medium containing 10% dialyzed fetal calf serum for 4 days.

In selected experiments low-density G_N-arrested cells, prepared as above, were refed complete DME medium containing 30% human platelet-poor-plasma-derived serum (PPP-S). These cells remained in the G_N arrest state and were designated G_N^+.

High-density G_N. This method produced proadipocytes G_N arrested at a density of $\approx 1 \times 10^5$ cells per cm^2. G_N cells, initially arrested at a low density, were trypsinized and replated at a final density of $\approx 1 \times 10^5$ cells per cm^2 and cultured in isoleucine-deficient DME medium/10% dialyzed fetal calf serum for 4 days.

Preparation of Human Blood Products. Human platelet-poor plasma and PPP-S were prepared essentially as described by Rutherford and Ross (13). Human plasma was fractionated by a modification of the barium citrate adsorption procedure of Mann (14) that will be reported in detail elsewhere. Three plasma fractions were obtained: barium citrate-adsorbed plasma (BaP), a citrate eluate of plasma components that adsorbed to barium citrate (CEP), and an EDTA eluate of plasma components that adsorbed to barium citrate (EDTAP). These fractions were used at a final concentration comparable to 25% complete plasma. The BaP fraction induces G_D arrest but does not induce adipocyte differentiation. The CEP fraction and a combination of the CEP and EDTAP fractions induce proadipocytes to differentiate but only if they have been previously G_D arrested.

RESULTS

Differentiation of Proadipocytes. Fig. 1 illustrates the kinetics of G_D arrest and differentiation in high-density cultures. Addition of fetal calf serum and insulin to confluent cells induces at least one round of cell division; 90% of the cells incorporate [^{3}H]thymidine into DNA and the cell density increases from $\approx 5 \times 10^4$ to $\approx 1 \times 10^5$ cells per cm^2. Thereafter, 90% of the cells become arrested in the G_1 phase of the cell cycle at G_D (Table 1) and remain at this state even after subsequent feedings. Maximal differentiation occurs after culture for 16–18 days. Fig. 2 shows the close correlation that exists between morphological differentiation and the increased activity of lipoprotein lipase and glycerol-3-phosphate dehydrogenase.

The kinetics of G_D arrest and differentiation of low-density proadipocytes in HHP are presented in Table 2. G_1 growth arrest of >90% of the cells at G_D occurs within 2–3 days (Table 1). G_D-arrested cells thereafter parasynchronously differentiate in the absence of significant mitogenesis. The extent of adipocyte differentiation in HHP approaches 100% (Fig. 2).

Relative Differentiation Potential of G_D- and G_S-Arrested Cells. The relative ability of G_D- and G_S-arrested cells to differentiate was assayed. G_S-arrested cells could not be exposed to differentiation-promoting medium containing high concentrations of serum and insulin because it is mitogenic for G_S cells. Therefore, nonmitogenic PPP-S containing insulin (50 μg/ml), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 100 ng/ml), or both or heparinized medium containing 25% nonmitogenic human plasma (HHP) was used. Table 3 shows that these media promote the differentiation of high-density G_D-arrested cells but do not promote the differentiation of confluent G_S cells. PPP-S + insulin and HHP are not mitogenic for G_S-arrested cells, and cells in this medium stay G_S arrested as shown by their retention of mitogenic responsiveness to growth factors and their lack of mitogenic responsiveness to nutrients (data not shown).

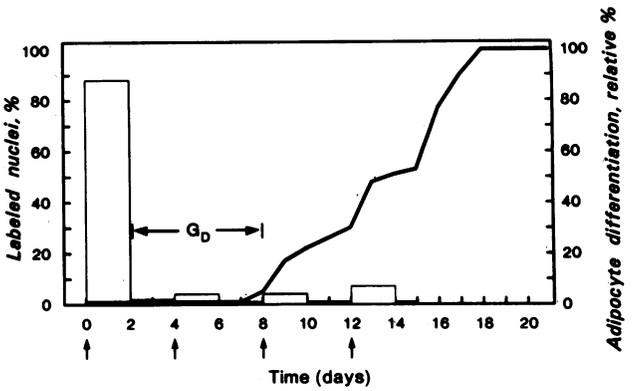

FIG. 1. Kinetics of G_D arrest and differentiation of high-density proadipocytes in fetal calf serum and insulin. Addition of high concentrations of serum and insulin to confluent 3T3 T cells induces >90% of the proadipocytes to traverse the cell cycle as determined by autoradiographic measurement of the incorporated [^{3}H]thymidine into DNA (bars). Thereafter the cells are mitogenically unresponsive to refeeding and are G_1 arrested. Six to eight days after G_1 growth arrest, the first morphological evidence of adipocyte differentiation developed (——). The interval between G_1 growth arrest and differentiation is designated G_D. One hundred percent relative adipocyte differentiation is equivalent to 40% absolute differentiation or 175 adipocyte foci per plate. The days on which the cells were refed fresh medium are indicated by arrows.

Because confluent G_S-arrested cells are only half the density of high-density G_D cells, we tested the effects of high density—i.e., $\approx 1 \times 10^5$ cells per cm^2—on the ability of G_S-arrested cells to differentiate. No differentiation of high-density G_S-arrested cells occurred when they were cultured in PPP-S supplemented with insulin, $PGF_{2\alpha}$ or both agents for intervals up to 21 days with repeated feedings on days 4, 8, and 12. HHP also failed to induce the differentiation of high-density G_S-arrested cells.

These data suggest that the G_D arrest state is distinct from the G_S arrest state. Data presented in Table 4 provide independent support for this conclusion. It shows that G_D-arrested

Table 1. Cell cycle analysis by flow microfluorimetry

Cells	Cell cycle distribution, %		
	G_1	S	G_2/M
Rapidly growing	54	38	8
G_S-arrested	92	5	3
G_N-arrested	80	14	6
G_D-arrested in fetal calf serum + insulin	92	4	4
G_D-arrested in HHP	96	2	2

Cells were prepared for analysis by flow microfluorimetry as described (15). Mithramycin (100 μg/ml) was employed as the DNA stain. Analyses were performed on a FACS IV instrument (Becton Dickinson, Sunnyvale, CA). The percent cells in different phases of the cell cycle was determined as described (15). HHP, heparinized medium containing human plasma.

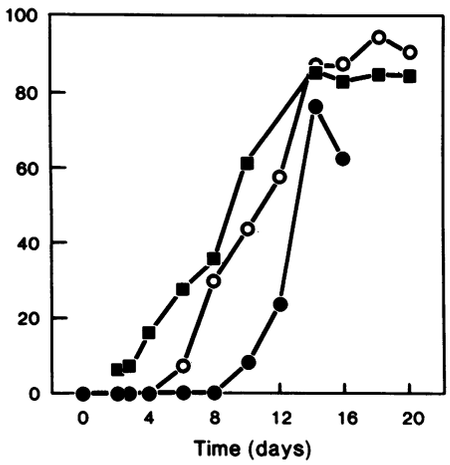

FIG. 2. Correlation between the kinetics of morphological and enzymatic differentiation. See the legend to Fig. 1. The ordinate indicates relative percent differentiated cells (○); lipoprotein lipase activity, % hydrolysis per 10^6 cells (□); and glycerol-3-phosphate dehydrogenase activity, μmol of NADH oxidized × 10^{-2} per min per mg of protein (●).

cells are responsive to the mitogenic effect of 3-isobutyl-1-methylxanthine; G_S-arrested cells are not. This is true irrespective of the cell density at which G_D or G_S cells are assayed.

Relative Differentiation Potential of G_D- and G_N-Arrested Cells. Six different nutrient-deficient media containing dialyzed fetal calf serum were initially screened for their ability to induce G_1 arrest of proadipocytes. These media were deficient in PO_4 (16), glutamine and histidine (16, 17), cysteine and methionine (16–18), 99% of all amino acids (17, 18), seven selected amino acids and glucose (19), or isoleucine (12). Only isoleucine-deficient medium containing dialyzed 10% fetal calf serum both induced G_1 arrest and supported proadipocyte viability for the 21-day interval required to perform these experiments. It was, therefore, used to induce G_N arrest in this study.

Table 1 shows that culture of proadipocytes in isoleucine-deficient medium for three days causes 80% of the cells to become arrested in G_1. Approximately 20% of isoleucine-arrested cells are in S (20).

To determine if G_N-arrested cells can differentiate in the absence of DNA synthesis, cells were cultured in the presence of isoleucine-deficient DME medium containing 30% dialyzed fetal calf serum ± insulin. Table 5 shows that no differentiation is observed under these conditions. G_N-arrested cells also fail to differentiate when cultured in isoleucine-deficient DME medium containing dialyzed 30% nonmitogenic PPP-S ± insulin, $PGF_{2\alpha}$, or both additives.

It was possible that omission of isoleucine from the culture medium inhibited expression of the differentiated phenotype,

Table 2. Kinetics of proadipocyte differentiation in HHP

Time after addition of HHP, days	Labeled nuclei,* %	Adipocyte differentiation, %	G_D arrest
0	100	0	−
2	96	0	+
4	10	5	+
6	4	78	−
8	4	90	−
10	2	96	−
12	1	97	−

* Determined autoradiographically by incubation in [^{3}H]thymidine for the 48 hr prior to the day indicated.

Table 3. Comparative differentiation of G_D- and G_S-arrested cells

Medium additives	Adipocyte differentiation, % G_D	G_S
30% fetal calf serum + insulin	100	—
30% PPP-S	0	0
30% PPP-S + insulin	40	0
30% PPP-S + $PGF_{2\alpha}$	25	0
30% PPP-S + insulin + $PGF_{2\alpha}$	50	0
25% HHP	100	6
10% fetal calf serum	0	0

G_D cells were at high density and G_S cells were confluent. The maximal differentiation in medium containing 30% fetal calf serum + insulin is designated 100%.

so we developed an isoleucine-rich differentiation-promoting medium that maintains the cells in G_1. Specifically, G_N-arrested proadipocytes were placed in complete DME medium containing PPP-S ± insulin, $PGF_{2\alpha}$ or both additives. These cells were designated G_N^+. In this medium cells do not enter the S phase of the cell cycle and no differentiation of G_N^+-arrested cells was observed even when they were repeatedly fed at 4-day intervals for 12 days and observed for 3 weeks.

In the above studies, G_N-arrested cells were not arrested at the same density as G_D-arrested cells. We, therefore, prepared high-density G_N-arrested cells (see *Materials and Methods*) and showed that they are also incapable of expressing the adipocyte phenotype in 30% PPP-S + insulin, $PGF_{2\alpha}$, or both (data not shown). This shows that cells arrested at G_N lack the capacity to differentiate in the presence of complete differentiation-promoting medium.

Effect of Prior Culture of G_S-Arrested Cells in Differentiation-Promoting Medium on their Potential to Differentiate. Differentiation of some cells requires the traverse of at least one complete cell cycle in the presence of differentiation-promoting medium. Proadipocytes were therefore first G_D arrested by culture in heparinized DME medium containing 25% plasma or barium citrate-adsorbed plasma for 4 days (see *Materials and Methods*). Flow microfluorimetry showed that >90% of these cells were G_1 arrested at G_D (Table 6A). G_D-arrested cells were mitogenically responsive to isobutylmethylxanthine and differentiated in the absence of DNA synthesis when CEP and EDTAP was added.

If G_D-arrested cells were cultured in DME medium con-

Table 4. Mitogenic response of G_1-arrested proadipocytes to isobutylmethylxanthine

Cells	Isobutylmethylxanthine-induced mitogenesis,* %
G_D-arrested	
High-density	43
Low-density	45
G_S-arrested	
High-density	8
Low-density	7
G_N-arrested	
Low-density	7

* Mitogenic responsiveness to isobutylmethylxanthine (0.5 mM) was determined by autoradiographic analysis of [^{3}H]thymidine incorporation into DNA for 48 hr. The data, expressed as the percent labeled nuclei in isobutylmethylxanthine-treated specimens minus that observed in untreated specimens, represent the mean values of more than six experiments. The standard error of the mean was less than 10%.

Table 5. Comparative differentiation of G_D- and G_N-arrested cells

Medium additives	Adipocyte differentiation, %		
	G_D	G_N	G_N^+
30% fetal calf serum + insulin	100	0	—
30% PPP-S	0	0	0
30% PPP-S + insulin	40	0	0
30% PPP-S + $PGF_{2\alpha}$	25	0	0
30% PPP-S + insulin + $PGF_{2\alpha}$	50	0	0

G_D cells were at high density and G_N cells were at low density. The designation G_N indicates that assays were performed in isoleucine-deficient DME medium containing dialyzed fetal calf serum. The designation G_N^+ indicates that assays were performed in complete DME medium and nondialyzed PPP-S. The percent differentiation observed in high-density G_D-arrested cells in medium containing 30% fetal calf serum and insulin was designated 100%. All media were nonmitogenic as determined by autoradiographic analysis except 30% PPP-S + insulin, which was mitogenic for 20–25% of G_N^+ cells.

taining 0.5% fetal calf serum for 3 days, they converted from the G_D to the G_S arrest state as evidenced by the fact that they become mitogenically unresponsive to isobutylmethylxanthine (Table 6*B*). Conversion from G_D to G_S was achieved in the absence of DNA synthesis.

If such G_S arrested cells were placed in heparinized medium containing CEP and EDTAP, no differentiation occurred and the cells remained at the G_S arrest state. This establishes that G_S-arrested cells cannot differentiate even after critical mitosis in differentiation-promoting medium.

Numerous studies also attempted to arrest cells in G_N after prior culture in differentiation-promoting medium. It was, however, not possible to efficiently capture cells at G_N after release from G_D because a significant fraction of the cells always entered the S phase of the cell cycle.

DISCUSSION

The coupling of proadipocyte growth arrest and differentiation is shown to occur at a distinct state in the G_1 phase of the cell cycle, G_D. Cells arrested at G_D are capable of expressing the differentiated adipocyte phenotype in the absence of DNA synthesis, whereas cells G_1-arrested at other states by growth factor or serum deprivation (G_S) or by nutrient deprivation (G_N) are unable to differentiate under nonmitogenic differentiation-promoting conditions. Moreover, prior culture of G_S-arrested cells in differentiation-promoting medium does not confer on such cells the capacity to differentiate. Therefore, exposure of cells to differentiation-promoting medium *per se* is not sufficient to induce differentiation. Rather, cells must be arrested and maintained at the G_D state in order for differentiation to occur.

Table 6. Effect of prior culture of G_S cells in differentiation-promoting medium on their potential to differentiate

Medium	Differentiation, %	Isobutylmethylxanthine response, %	Labeled nuclei, %
A. G_D-arrested cells			
BaP	5	36	4
BaP + CEP and EDTAP	42	32	1
B. G_S-arrested cells			
BaP → 0.5% fetal calf serum	0	2	4
BaP → 0.5% fetal calf serum + CEP and EDTAP	6	4	3

Differentiation was assayed 20 days after growth arrest. The mitogenic responsiveness of G_D- and G_S-arrested cells to isobutylmethylxanthine at 0.5 mM was determined as in Table 5. The percent labeled nuclei indicates the total number of labeled cells from the time cells were placed in a designated condition until 20 days later.

By a totally independent method we also established that G_D-arrested cells are distinct from G_S- and G_N-arrested cells. G_D-arrested cells are shown to be sensitive to a mitogenic effect of isobutylmethylxanthine, whereas G_S- and G_N-arrested cells are not. Although the mechanism of action of isobutylmethylxanthine on G_D-arrested cells is not known, the data establish that G_D arrest is reversible. Reversible G_1 arrest was also reported to precede differentiation of L_6 myoblasts (4).

The coupling of proadipocyte growth arrest and differentiation, therefore, has been shown to occur at G_D. Cells growth arrested at this state mediate the coupling process by their ability to either differentiate or reinitiate cell proliferation. We suggest that the coupling of growth arrest and differentiation at G_D is an important physiological regulator of cell proliferation because it appears to mimic the regulatory events that control cell proliferation *in vivo* (1–2).

Previous attempts to identify the physiological basis of normal growth control have also focused on events that occur in the G_1 phase of the cell cycle. These results have been interpreted to show that there are specific restriction points in the G_1 phase that control proliferation (21), perhaps by probabilistic kinetics (22). Deprivation of growth factors or low molecular weight nutrients has been implicated in the mediation of these growth arrest processes.

The physiological significance of growth control regulated by growth factor or nutrient deprivation must, however, be questioned because there is insufficient data to support the conclusion that cell growth *in vivo* is regulated by comparable processes. The available evidence rather suggests that cells *in vivo* regulate their growth rate by expressing a differentiated phenotype. If physiologically significant data are to be obtained concerning the control of cell proliferation, we suggest that cells with the capacity to differentiate must be studied. Using proadipocytes that can differentiate into fat cells, we have shown in this paper that cell proliferation can be mediated by the coupling of growth arrest and differentiation at a distinct state in the cell cycle. We suggest that growth control *in vivo* may also occur at a state comparable to G_D.

Previous studies have also attempted to identify defects in growth control in malignant cells. These studies have employed virally or chemically transformed cells derived from undifferentiated mouse embryo cell lines. It has been suggested that such transformed cells lack the ability to become G_S arrested after growth factor deprivation and that this characteristic might result from endogenous growth factor production by these transformed cells (23, 24). The failure of transformed cells to become arrested at G_S has been proposed to be one explanation why malignant cells grow rapidly and produce tumors.

Rapid growth is, however, not a general characteristic of transformed cells. Many malignant cells *in vivo* do not grow rapidly. In fact, many malignant tumors grow slowly and express differentiated characteristics (25). We suggest that in order to identify the mechanism of action of carcinogens it is essential to study their effects on cells with the capacity to differentiate. In such systems we suggest that malignant transformation will be found to result from defective coupling of growth and differentiation, and we have preliminary data to support this hypothesis.

We have studied many clones of transformed 3T3 T proad-

ipocytes and have found that they can maintain their ability to arrest in G_S and G_N and still be tumorigenic. All transformed tumorigenic 3T3 T clones by contrast lack the ability to couple the control of growth and differentiation at G_D (unpublished data). We have also obtained preliminary data that UV irradiation, at doses that can initiate carcinogenesis, blocks the ability of proadipocytes to become arrested in G_D and differentiate (unpublished data). These observations support the conclusion that defects in the coupling of growth arrest and differentiation at G_D may occur in the early stages of malignant transformation.

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