

# Coupling of Preadipocyte Growth Arrest and Differentiation.

## II. A Cell Cycle Model for the Physiological Control of Cell Proliferation

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**ABSTRACT** Experimental evidence is presented that supports a cell cycle model showing that there are five distinct biological processes involved in preadipocyte differentiation. These include: (a) growth arrest at a distinct state in the  $G_1$  phase of the cell cycle; (b) nonterminal differentiation; (c) terminal differentiation; (d) loss of the differentiated phenotype; and (e) reinitiation of cell proliferation. Each of these events is shown to be regulated by specific human plasma components or other physiological factors. At two states designated  $G_D$  and  $G_{D'}$ , coupling of growth arrest and differentiation is shown to occur. We propose that these mechanisms for the coupling of growth arrest and differentiation are physiologically significant and mimic the regulatory processes that control stem cell proliferation *in vivo*.

Cell proliferation *in vivo* and *in vitro* can be regulated by the coupling of growth arrest and cell differentiation (7, 8, 11, 12, 16). The mechanisms that control the coupling process have been studied using BALB/c 3T3 T preadipocytes which differentiate into fat cells in culture (5, 10, 16). We established that, before expression of the adipocyte phenotype, growth arrest must occur at a state in the  $G_1$  phase of the cell cycle, designated  $G_D$ , and that  $G_D$  is distinct from other  $G_1$  growth arrest states, including those induced by density-dependent growth inhibition, growth factor deprivation, and nutrient deprivation (16). Only  $G_D$ -arrested cells: (a) can express the differentiated phenotype in the absence of DNA synthesis (16); (b) can be stimulated to proliferate when exposed to methyl isobutyl xanthine (16); and (c) are sensitive to a cytotoxic effect of 8-bromo cyclic AMP (6). The  $G_D$  arrest state is also distinct from other  $G_1$  states on the basis of topographical analysis (19). In previous studies, however, it has been difficult to distinguish other possible stages in the differentiation process because of technical limitations.

In the preceding paper (10) we described a new culture system that induces preadipocyte  $G_D$  arrest and differentiation. It was established that culture of low density preadipocytes in heparinized medium containing human plasma induces  $G_D$  arrest and differentiation in a rapid, highly efficient, and parasynchronous manner. With this method it is now possible to perform more detailed studies on the mechanisms that

regulate the coupling process. Data are presented in this paper which provide the basis for a new model that shows that five distinct biological processes are involved in preadipocyte differentiation and that coupling of growth arrest and differentiation occurs at two states, designated  $G_D$  and  $G_{D'}$ .

### MATERIALS AND METHODS

#### General

The BALB/3T3 T preadipocyte cell line was used in this study. To induce  $G_D$  arrest and differentiation, 3T3 T cells were cultured in heparinized Dulbecco's modified Eagle's medium (DME) containing either 25% human plasma or human plasma fractions as described (10). The methods used to measure [ $^3$ H]thymidine incorporation by autoradiography and to perform flow microfluorimetric analysis of the DNA content to characterize cell cycle distribution have been previously described (10, 16), as have the assays used to determine morphological differentiation (10, 16). The activity of glycerol-3-phosphate dehydrogenase (9) was also used as an assay for adipocyte differentiation, because it has recently been shown that, under selected culture conditions, cells can undergo enzymatic differentiation in the absence of morphological differentiation (17). The density of undifferentiated and differentiated cells was quantitated by microscopic analysis using calibrated eyepieces; cell counts assayed between 300 and 500 cells for each specimen.

#### Preparation of Human Plasma Fractions

Citrate anticoagulated platelet-poor human plasma was prepared from human blood obtained by venipuncture as described (10). It was then fractionated by either of the two methods. The first method of human plasma fractionation was

previously described in detail (10). Briefly, citrate anticoagulated plasma was adsorbed repeatedly with barium chloride. This produced barium adsorbed plasma (BaP) and plasma components bound to the barium citrate precipitate. Sequential elution of the barium adsorbed plasma components with sodium citrate and ethylenediamine tetraacetic acid produced two fractions designated CEP and EDTAP, respectively.

A second method was developed to produce another fraction with different biological activity. With this method the pH at which the barium adsorption was performed was changed from pH 7.8 to 8.7. More specifically, citrate anticoagulated human plasma was adjusted to pH 8.7 with 1 N NaOH; barium chloride was then added slowly to a final concentration of 0.1 M. The pH was readjusted to 8.7 and continually stirred at 4°C for 30 min. The suspension was then centrifuged at 3600 g for 25 min at 4°C. The sediment was finally partially solubilized by addition of 0.9% NaCl-0.02 M sodium citrate. This suspension was stirred for 45 min at 4°C and then sedimented as above. The supernatant, which was extensively dialyzed against 6.5 mM sodium citrate-citric acid buffer (pH 7.4), was designated CEPH. Other methods of procedure are described in detail in the preceding paper (10).

## RESULTS

The culture of rapidly growing low density proadipocytes in heparinized medium containing human plasma induces growth arrest and differentiation. The studies described in this paper were designed to establish: (a) if reversible growth arrest at a distinct state in the  $G_1$  phase of the cell cycle precedes differentiation; (b) if the differentiation process involves nonterminal and terminal phases; (c) if nonterminally differentiated cells can be induced to dedifferentiate; and (d) if  $G_D$ -arrested and dedifferentiated cells can be induced to reinitiate cell proliferation.

### Growth Arrest at the $G_D$ State

The culture of proadipocytes in heparinized medium containing human plasma induces growth arrest at a distinct state in the  $G_1$  phase of the cell cycle,  $G_D$ , and their subsequent differentiation (10, 16). In order to further establish that  $G_D$  is a specific phase in the coupling process, we attempted to identify experimental conditions that would induce and maintain cells in the  $G_D$  arrest state in the absence of differentiation and in the absence of DNA synthesis. Three criteria were used to establish that cells were at the  $G_D$  state. First, cells had to have a 2 N DNA content, which is characteristic of cells growth arrested in the  $G_1$  phase of the cell cycle. Second, cells had to show mitogenic responsiveness to methyl isobutyl xanthine (MIX) (16). Third, the cells had to be capable of expressing the adipocyte phenotype without DNA synthesis (16). The latter two biological characteristics distinguish  $G_D$  arrested cells from cells arrested at other states in the  $G_1$  phase of the cell cycle (16).

Since adsorption of citrated human plasma with barium chloride removes its differentiation-promoting activity, we tested to determine if the culture of proadipocytes in heparinized medium containing barium adsorbed plasma (BaP) induces cells to growth arrest at  $G_D$  without subsequently expressing a differentiated phenotype. The data presented in Tables I-III support this possibility. Table I shows that culture of proadipocytes in heparinized medium containing BaP induces the growth arrest in >90% of the cells in the  $G_1$  phase of the cell cycle. Growth arrest under these culture conditions occurs within 3 d, and the kinetics of growth arrest assayed by decreased incorporation of [ $^3$ H]thymidine into DNA were comparable to those observed when proadipocytes were cultured in heparinized DME containing complete human plasma. We also observed that cells could be maintained at  $G_D$  in this medium for intervals as long as 2 wk without loss of viability and without loss of the  $G_D$  associated characteristics described

TABLE I  
Induction of Growth Arrest of Proadipocytes in the  $G_1$  Phase of the Cell Cycle by Culture in Heparinized Medium Containing Barium Adsorbed Human Plasma

	Cell cycle distribution		
	$G_1$	S	$G_2/M$
	%		
Rapidly growing cells	54	24	22
Cells growth-arrested in BaP	93	3	4

The distribution of cell populations in various phases of the cell cycle was determined by flow microfluorography using a FACS IV apparatus (see Materials and Methods). BaP designates heparinized DME containing 25% barium adsorbed human plasma. Cells were assayed during exponential growth or after culture in BaP for either 4 or 8 d. Comparable results were obtained for both specimens cultured in heparinized medium containing BaP.

TABLE II  
Mitogenic Responsiveness to Methyl Isobutyl Xanthine of Proadipocytes Growth-Arrested in Heparinized Medium Containing Barium Adsorbed Human Plasma

Growth arrest medium supplement	Labeled nuclei
	%
0.5% FCS	5
25% BaP	46
25% HP	42

Cells were growth-arrested by culture in DME-0.5% fetal calf serum (FCS) for 4-6 d or by culture in heparinized DME containing either barium adsorbed human plasma (BaP) or complete human plasma (HP) for 4 to 8 d. To the culture medium was then added methyl isobutyl xanthine, final concentration  $5 \times 10^{-4}$  M, and [ $^3$ H]thymidine. Cells were incubated in this medium for 48 h and were then fixed and processed for autoradiographic analysis to determine the extent of DNA synthesis. The data represent the percent labeled nuclei in MIX-treated cells minus the percent labeled nuclei in untreated cells.

TABLE III  
Induction of Differentiation of Cells  $G_D$ -Arrested in Heparinized Medium Containing Barium Adsorbed Human Plasma

Treatment	Adipocyte differentiation		Labeled nuclei
	%	Fat cells/cm <sup>2</sup>	%
BaP alone	5	23	1
BaP $\times$ 4 d, then CEP	52	280	6
BaP $\times$ 8 d, then CEP	65	325	3

Cells were cultured in heparinized medium containing BaP for 4 or 8 d. CEP was then added together with [ $^3$ H]thymidine and the cells were cultured for 12 additional days; at 4-d intervals cultures were fed fresh heparinized medium containing BaP or BaP and CEP in addition to [ $^3$ H]thymidine. The cells were then fixed in formaldehyde-glutaraldehyde and prepared for autoradiography. The extent of adipocyte differentiation was assayed both before and after autoradiography with comparable results. The percent labeled nuclei indicated includes all cells that incorporate [ $^3$ H]thymidine from the time that CEP was added until termination of the experiment.

in Tables II and III. These characteristics included mitogenic responsiveness to MIX and the capacity to differentiate without DNA synthesis. The data in Table II show that cells growth-arrested by culture in heparinized medium containing BaP respond to the mitogenic effect of MIX in a manner similar to cells  $G_D$ -arrested by culture in heparinized medium containing complete human plasma. This is in contrast to cells growth-arrested in  $G_1$  following serum deprivation; such cells are not sensitive to MIX-induced mitogenesis.

The data in Table III provide additional support for the conclusion that proadipocytes can be arrested and maintained

in the differentiation-competent  $G_D$  arrest state by culture in heparinized medium containing BaP. The data show that after addition of CEP to cells  $G_1$  growth-arrested in heparinized medium containing BaP, differentiation proceeds without DNA synthesis. We interpret these data to support the conclusion that  $G_D$  arrest and differentiation are separable events.

### Nonterminal and Terminal Differentiation

Studies were next performed to determine if the differentiation of proadipocytes is a terminal or a nonterminal process. We first assayed whether adipocyte differentiation in heparinized DME containing human plasma is tightly coupled to the loss of proliferative capacity. The data in Fig. 1 show that there is approximately a 3-d lag between expression of the adipocyte phenotype and loss of proliferative potential, i.e., terminal differentiation. This suggests that a stage of nonterminal differentiation must exist. For example, Fig. 1 shows that on the sixth day, when approximately 50% of the cells are differentiated, 95% can be induced to incorporate [ $^3$ H]thymidine into DNA. However, after the 12th day, minimal proliferation of differentiated cells can be detected.

To substantiate the possibility that there is a nonterminal phase of adipocyte differentiation, we attempted to identify experimental conditions that would limit the terminal phase of differentiation and thereby enrich cultures in nonterminally differentiated cells. We found that the culture of low density cells in heparinized DME containing a plasma fraction designated CEPH achieved this result. Table IV presents data on the relative ability of human plasma, BaP, and CEPH to induce adipocyte differentiation. It shows that heparinized medium containing only CEPH can induce differentiation comparable to that observed in heparinized medium containing whole plasma. The culture of proadipocytes in medium containing CEPH was also found to induce  $G_1$  growth arrest. Flow microfluorimetric analysis of such cells showed the following cell cycle distribution:  $G_1$ , 87%; S, 3%;  $G_2/M$ , 10%.

The data in Table V establish that heparinized DME containing CEPH selectively induces nonterminal adipocyte differentiation at a state we have designated  $G_D'$ . If cells are cultured in heparinized DME containing CEPH for 8 d or longer, they differentiate but can be induced to lose the differentiated phenotype when exposed to retinoic acid or other

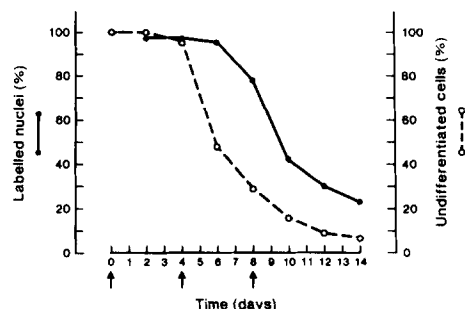


FIGURE 1 Nonterminal and terminal phases of adipocyte differentiation. Low density proadipocytes were cultured in heparinized DME containing human plasma. Periodically, the cells were assayed for their extent of differentiation and for their ability to proliferate. For the latter assays, cells were refed DME containing 30% FCS and [ $^3$ H]thymidine and the percent of the cells that synthesized DNA in the subsequent 48 h was determined autoradiographically. The data show that adipocyte differentiation occurs before the loss of proliferative potential.

TABLE IV

Differentiation of Proadipocytes Cultured in Heparinized Medium Containing Different Human Plasma Fractions

Additives to heparinized DME	Adipocyte differentiation %
HP	65-95
CEPH	50-75
BaP	5-10
CEP	0-10
BaP and CEP	50-70
Serum	0

Low density proadipocytes were cultured in heparinized DME containing the designated plasma fractions at concentrations calculated to be comparable to 25% whole plasma or in DME containing 10% FCS. Cells were fed these media at 4-d intervals for 12 d and were assayed to the extent of morphological adipocyte differentiation on the 15th day. The results are given as the range in the percent adipocyte differentiation observed in three or more experiments with the indicated plasma fractions. HP designates whole human plasma; the description of the other plasma fractions are detailed in Materials and Methods.

TABLE V

Nonterminal Differentiation Resulting from Culture of Proadipocytes in Heparinized Medium Containing a Plasma Fraction Designated CEPH

Heparinized DME additives	Other additives	Differentiated cells Adipocytes/cm <sup>2</sup>	Loss of the differentiated phenotype %
HP		2,350	
HP	Retinoic acid	2,525	0
CEPH		1,545	
CEPH	Retinoic acid	513	67

Low density cells were cultured in heparinized DME containing either 25% human plasma (HP) or CEPH for 12 d with refeeding at 4-d intervals. They showed comparable differentiation kinetics. Retinoic acid (10-20  $\mu$ g/ml) was then added on the 12th day to one-half of the flasks and the quantity of fat cells present was determined on the 15th day. During this interval a gradual loss of the adipocyte phenotype was morphologically evident in CEPH-treated cells. Since retinoic acid is cytotoxic to some cells, the data have been corrected for maximum potential cytotoxicity. Comparable results were obtained in two separate experiments.

select drugs; they can also be induced to synthesize DNA if subsequently fed 30% fetal calf serum (FCS) (Table V and Fig. 2A). By contrast, retinoic acid does not induce significant numbers of cells cultured in heparinized DME containing complete human plasma for 10-12 d to lose the differentiated phenotype. Addition of mitogens, such as 30% serum, also does not induce the latter cells to incorporate [ $^3$ H]thymidine (Fig. 1).

These observations suggest that nonterminal and terminal adipocyte differentiation are distinct phases in the coupling process and that CEPH lacks the plasma components required for terminal differentiation. The data presented in Table VI support this conclusion. They show that nonterminally differentiated cells cultured in heparinized medium containing CEPH can be converted to a terminally differentiated state by addition of complete human plasma, or the plasma fractions designated BaP or EDTAP, but not by CEP.

## Loss of the Differentiated Phenotype

The data reported in Table V and Fig. 2 show that nonterminally differentiated cells can be induced to lose the differentiated phenotype and reinitiate cell proliferation. These two events are separable processes because the data show that, although 67% of nonterminally differentiated cells cultured in heparinized medium containing CEPH can be induced to lose the differentiated phenotype during a 3-d interval in retinoic acid, <1% of the cells incorporate [<sup>3</sup>H]thymidine into DNA during this interval. Reinitiation of proliferation of cells induced to dedifferentiate by retinoic acid requires treatment with serum or other mitogenic agents (Fig. 2). Loss of the differentiated phenotype and reinitiation of proliferation are, therefore, separate phases in the coupling process. Under selected experimental conditions it is also possible to induce nonterminally differentiated adipocytes to proliferate without loss of the differentiated phenotype (data not shown).

## Reinitiation of Cell Proliferation

Cells which have G<sub>D</sub>-arrested but which have not expressed the adipocyte phenotype can also be induced to reinitiate DNA synthesis and traverse the cell cycle by addition of a variety of mitogens directly to the differentiation-promoting medium. The data in Table VII list these mitogens. They include epidermal growth factor, phorbol myristate acetate, methyl isobutyl xanthine, 30% fetal calf serum, and a cyst fluid, designated TuGF, which was derived from tumors induced by implantation of BALB/c 3T3 cells on plastic plates into syngeneic mice (4, 15).

TABLE VI  
Requirements for Specific Plasma Factors to Induce Terminal Adipocyte Differentiation

Heparinized DME additives	Loss of the adipocyte phenotype			
	Condition A		Condition B	
	Control	Treated	%	%
	<i>Adipocytes/cm<sup>2</sup></i>			
CEPH	1,875	600	68	70
CEPH and CEP				68
CEPH and BaP	2,375	1,500	37	0
CEPH and HP	2,200	1,850	16	0
CEPH and EDTAP	2,775	2,125	23	

Condition A: Low density cells were cultured in heparinized DME containing 25% CEPH for 12 d with prior feedings at 4-d intervals. On the 12th day an aliquot of complete human plasma (HP), barium adsorbed human plasma (BaP), or the EDTA eluate of barium adsorbed plasma components (EDTAP) was added. The concentration of these additives was equivalent to that calculated to be present in 25% HP. The ability of the adipocytes, cultured under various conditions, to lose the differentiated phenotypes was tested 3-d later by removal of the differentiation-promoting medium and culture of the cells in DME containing 30% fetal calf serum. Loss of the differentiated phenotype is expressed as the percent decrease in the density of fat cells. It was determined by analysis of the number of fat cells/cm<sup>2</sup> present before (control) and 3-d after addition of DME-FCS (treated). Cell density analysis showed that loss of the differentiated phenotypes was not associated with any cytotoxicity. Condition B: Comparable results were obtained when retinoic acid (10–20 µg/ml) was used to induce loss of the adipocyte phenotype. In these studies low density cells were cultured in heparinized medium containing CEPH for 8 d with feeding at 4-d intervals. On the eighth day the cultures were refed medium containing either HP, BaP or CEP. On the 12th day retinoic acid was added, and loss of the differentiated phenotype was scored 3-d later. Retinoic acid caused some cytotoxicity under these culture conditions. Cytotoxicity was, however, found not to account totally for loss of the adipocyte phenotype. Even if the assumption is made that cytotoxicity affected only differentiated cells, calculations established that retinoic acid induced >20% of adipocytes to lose the differentiated phenotype.

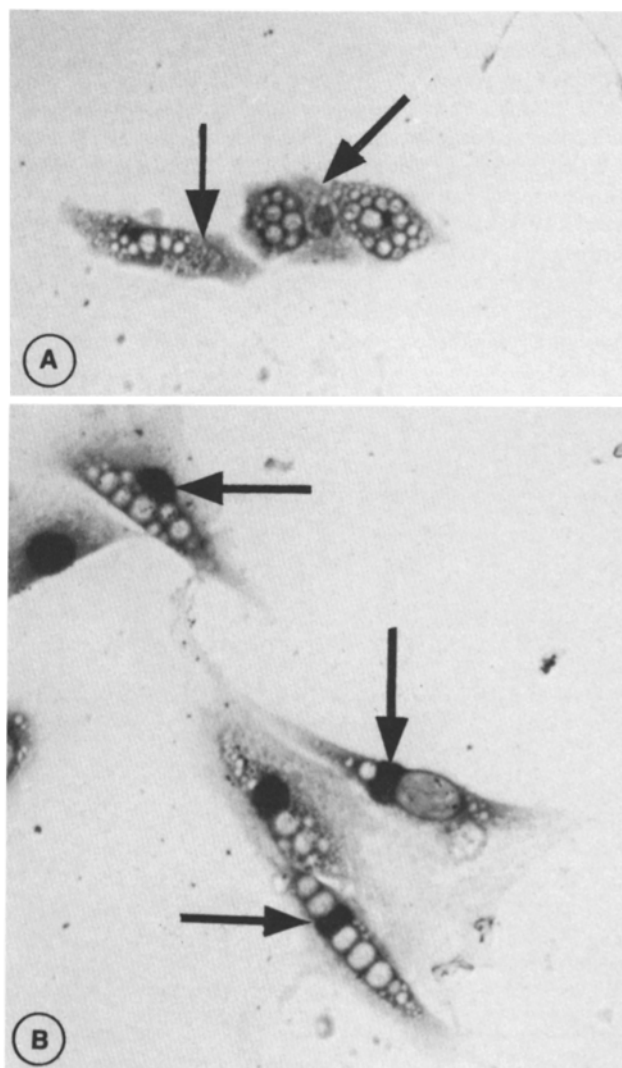


FIGURE 2 Nonterminal adipocyte differentiation: morphological evidence that adipocytes can undergo a proliferative response. Low density cells were cultured for 8 d in heparinized DME containing the plasma fraction designated CEPH to induce nonterminal adipocyte differentiation. They were then maintained in this medium for an additional 3 d with or without (*trans*) retinoic acid (RA). They were subsequently fed 30% FCS and [<sup>3</sup>H]thymidine. Cells maintained in medium containing CEPH remained differentiated and did not undergo DNA synthesis (A), whereas many cells treated with RA lost their adipocyte phenotype and incorporated [<sup>3</sup>H]thymidine into DNA during the subsequent 3-d incubation interval in 30% serum containing medium (B). Some adipocytes, such as those illustrated, became labeled before all of their intracellular triglyceride disappeared. The results of this and related studies established that the minimum quantity of adipocytes that can lose the differentiated phenotype and proliferate in such experiments is 26–37%; the maximum number is ~70%. This range results from the fact that in some cases it is difficult to determine the difference between an undifferentiated cell and a differentiated cell that has been induced to dedifferentiate. Arrows mark nuclei within the cells. In (A) the nuclei are unlabeled, whereas in (B) many [<sup>3</sup>H]thymidine labeled nuclei are evident.

## Correlation Between Morphological and Enzymatic Differentiation

Studies were also performed to establish that the morphological criteria for adipocyte differentiation used in this paper

correlate well with enzymatic differentiation assayed by increased activity of glycerol-3-phosphate dehydrogenase. These experiments were performed because of the possibility that under certain culture conditions adipocytes might fail to accumulate intracellular triglyceride and, therefore, appear undifferentiated, even though they were differentiated by the criteria of containing high levels of lipogenic enzymes (17). The data in table VIII show that there is an excellent correlation between morphological and enzymatic differentiation of cells at various phases of coupling processes. This further establishes the validity of data reported in this paper which show that the coupling of proadipocyte growth arrest and differentiation is a multistep process that is primarily mediated at two states, designated  $G_D$  and  $G_D'$ .

## DISCUSSION

These results show that there are at least five biological processes involved in the control of proadipocyte differentiation.

TABLE VII  
Reinitiation of Cell Proliferation of  $G_D$ -Arrested Cells

Mitogen	Labeled nuclei
	%
Epidermal growth factor (100 ng/ml)	70
Tumor growth factor (25%)	85
Phorbol myristate acetate (100 ng/ml)	50
Fetal calf serum (10 or 30%)	90
Methyl isobutyl xanthine ( $2.5-5.0 \times 10^{-4}$ M)	45

Proadipocytes  $G_D$ -arrested by culture at low density in heparinized DME containing 25% human plasma for 4 d were treated with the listed additives and [ $^3$ H]thymidine for 48 h. The percentage of cells that synthesize DNA during this interval was determined autoradiographically. The optimum mitogenic concentration for each mitogen is given parenthetically. In all experiments the mitogens were added directly to the heparinized medium containing human plasma. The labeling index for unstimulated cells was typically ~5%. The data are expressed as mean values of experimental specimens ( $n > 3$ ) minus control specimens.

TABLE VIII  
Correlation between Morphological and Enzymatic Adipocyte Differentiation

Growth or differentiated state	Morphological differentiation	Enzymatic differentiation: * glycerol-3-phosphate dehydrogenase activity
	% Fat cells	$\mu\text{mol NADH oxidized}/\text{min}/\text{mg protein} \times 10^{-2}$
Rapidly growing (3 d in serum)	0	0
$G_D$ -arrested (8 d in BaP)	5	0
$G_D$ -arrested (4 d in HP)	15	$10.3 \pm 0.5$
Differentiated (11 d in HP)	75	$241.2 \pm 5.3$
Nonterminally differentiated (8 d in CEPH)	50	$197.2 \pm 1.6$
Dedifferentiated (8 d in CEPH, 4 d in RA)	5	0

Column one lists the growth or differentiation state of the cells that were assayed. The duration of culture and the various media that were employed to derive these cells are given parenthetically.

\* The term "dedifferentiated" is used as an abbreviation to designate those nonterminally differentiated cells that have been induced to lose the adipocyte phenotype. The sensitivity of the enzyme assay permitted detection of activity greater than 400 pmol NADH oxidized/min/mg protein. Data are expressed as the mean of triplicate determinations plus or minus standard error of the mean.

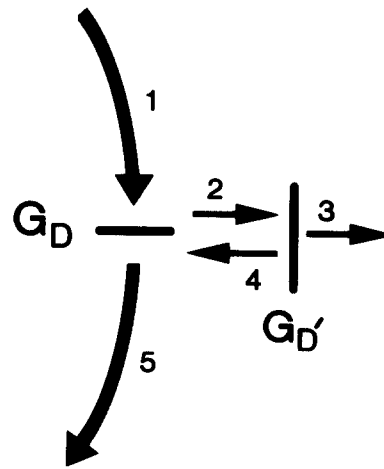


FIGURE 3 Model for the coupling of proadipocyte growth arrest and differentiation. Five phases are illustrated: Arrow 1), growth arrest at a distinct state in the  $G_1$  phase of the cell cycle,  $G_D$ ; arrow 2), nonterminal differentiation and arrest at  $G_D'$ ; arrow 3), terminal differentiation; arrow 4), loss of the differentiated phenotype; and arrow 5), reinitiation of cell proliferation. Regulation of

the coupling process is primarily mediated at  $G_D$  and  $G_D'$ , because  $G_D$ -arrested cells can either differentiate or reinitiate proliferation and  $G_D'$ -arrested cells can either terminally differentiate or reinitiate proliferation typically after loss of the differentiated phenotype.

Three processes promote differentiation and are regulated by physiological human plasma components. These include: (a) growth arrest at a distinct state in the  $G_1$  phase of the cell cycle; (b) nonterminal differentiation; and (c) terminal differentiation. Two processes antagonize the differentiation process. These include: (a) the reinitiation of proliferation of arrested cells; and (b) the dedifferentiation and proliferation of nonterminally differentiated cells. A schematic model for the relationship of these stages in the control of proadipocyte proliferation and differentiation is presented in Fig. 3.

The model illustrates that  $G_D$  and  $G_D'$  are states at which coupling of the control of cell proliferation and differentiation can be mediated. Cells growth-arrested at  $G_D$ , for example, have three potentialities. They can remain  $G_D$ -arrested; they can be induced to reinitiate proliferation (Fig. 3, arrow 5); or they can be induced to nonterminally differentiate (Fig. 3, arrow 2). Nonterminally differentiated cells at the  $G_D'$  state also have three potentialities. They can remain nonterminally differentiated; they can dedifferentiate and/or reinitiate proliferation (Fig. 3, arrows 4 and 5); or they can terminally differentiate (Fig. 3, arrow 3).

It is our hypothesis that the coupling of proadipocyte growth arrest and differentiation mediated at  $G_D$  and  $G_D'$  mimics physiological growth regulation, because in vivo the control of stem cell proliferation is coupled to expression of a differentiated phenotype by apparently similar mechanisms (7, 8, 11). For example, in hematopoietic and gastrointestinal stem cells,  $G_1$  growth arrest has been shown to occur before differentiation (8, 11). In vivo stages of nonterminal and terminal differentiation have also been identified and it has been shown that the reinitiation of proliferation of such nonterminally differentiated cells can be preceded by a dedifferentiation phase. These latter results were obtained on studies of hepatocytes (1-3).

These observations provide support for the physiological relevance for our model for the coupling of growth arrest and differentiation. Although the coupling process may vary in other cell types, we suggest that our model for the coupling of growth arrest and differentiation will be more useful in explaining the pathogenesis of human disease states than previous models for growth control (13, 18). For example, our model for growth control can form the basis for the hypothesis that in vivo and in vitro cellular aging results from acceleration in the terminal differentiation process, which is regulated at  $G_D'$ , and

that neoplastic transformation results from defective coupling of growth arrest and differentiation mediated at  $G_{1D}$  or  $G_{1D}'$  (14, 20).

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