

Regulation of the Terminal Event in Cellular Differentiation: Biological Mechanisms of the Loss of Proliferative Potential

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Abstract. Human plasma has been demonstrated to contain factors that induce the sequential expression of nonterminal and terminal adipocyte differentiation in 3T3 T mesenchymal stem cells. We now report the development of methods for the isolation of purified populations of nonterminally differentiated cells and terminally differentiated cells, and we show that it is possible to experimentally induce transition from the nonterminal to the terminal state of differentiation. With this model system it is therefore now possible to examine the biological and molecular processes associated with the terminal event in differentiation, i.e., the irreversible loss of proliferative potential. In this regard, we demonstrate that transition from the nonterminal

to terminal state of differentiation is a complex metabolic process that consists of at least two steps and that this process can be triggered by pulse exposure to an inducer for ~12 h but that ~24–48 h is required for the process to be completed. The data also establish that induction of the terminal event in differentiation requires protein synthesis but not RNA and DNA synthesis. These and additional results suggest that loss of proliferative potential associated with the terminal event in cellular differentiation is a distinct regulatory process, and we suggest that defects in this regulatory process may be of etiological significance in the pathogenesis of specific human diseases, especially cancer.

THE differentiation of most adult stem cells is associated with loss of proliferative capacity, i.e., terminal differentiation (27, 44). Epithelial stem cells have been clearly established to undergo terminal differentiation (49) as have hematopoietic, muscle, and neural cells (16). Even in differentiated hepatocytes and lymphocytes that retain the ability to divide (17, 23), loss of proliferative potential associated with the terminal event in differentiation has been documented; for example, lymphocytes can become terminally differentiated plasma cells (51). The process by which cells lose the ability to traverse the cell cycle and proliferate in association with induction of the terminal event in differentiation is an important physiological mechanism involved in tissue renewal and growth control. It may also represent a critical regulatory process that prevents the development of certain disease states, such as cancer (47).

Previous attempts to study the process of terminal differentiation have used a variety of *in vitro* model systems. These have included myeloid and erythroleukemia cell lines (4, 6, 29, 32), myoblast cell lines (25), and cultured epidermal cells (11, 42, 49, 52). Despite extensive analysis, the mechanisms that specifically control the terminal event in differentiation and loss of proliferative potential have not been established. This is so, because in the experimental systems used in previous studies, it has not been possible to clearly distinguish those processes associated with differentiation *per se* and those

processes associated with loss of proliferative potential. We have therefore developed a model tissue culture system to study cells at nonterminal and terminal states of differentiation (18, 34, 35, 38, 41).

This paper further characterizes cells at the nonterminal and terminal states of differentiation and describes methods to purify cells at these states. This paper also examines the biological mechanisms that mediate the terminal event in differentiation. The results establish that the transition from the nonterminal to terminal state of differentiation involves at least two biological steps that require 24–48 h to be completely induced and that these processes can be blocked by protein synthesis inhibitors. These and additional data suggest that control of the terminal event in cellular differentiation represents a distinct regulatory process.

Materials and Methods

Cell Culture

The BALB/c 3T3 T mesenchymal stem cell line (8) and clonal derivatives of these cells were used in all studies. Stock cultures were maintained in Dulbecco's modified Eagle's medium (DME)¹ containing 10% heat-inactivated fetal calf

¹ *Abbreviations used in this paper:* CEPH, citrate eluate of barium-precipitated plasma at high pH; DME, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HP, heparinized platelet-poor human plasma; MIX, methyl isobutyl xanthine; RA, retinoic acid; TCA, trichloroacetic acid; TPA, 12-O-tetradecanoylphorbol-13-acetate.

serum (FCS) at 37°C in a 5% CO₂ atmosphere as previously described (18, 34, 35, 38, 41). All stock cultures of cells were maintained at low density in 75-cm² flasks by passage with 0.1% trypsin and 1 mM EDTA in phosphate-buffered saline (PBS) when cells were <60% confluent. This procedure was used to prevent the cultural selection of cells that can grow at high cell densities and thereby express a "transformed" phenotype. Cell cultures were also periodically assayed and proven to be free of mycoplasma contamination by the method of Chen (3).

Preparation of Human Plasma and Human Plasma Fractions

Preparation of human plasma and plasma fractions is described in more detail elsewhere (18). Briefly, platelet poor citrate anticoagulated plasma was obtained from normal volunteers by standard venipuncture or from patients undergoing plasmapheresis as therapy for autoimmune disorders. These plasma lots were frozen at -70°C. When needed, they were thawed at 4°C and centrifuged at 1,000 g for 10 min at 4°C before use. Such whole human plasma specimens are designated HP.

Whole human plasma was processed to prepare a fraction that preferentially induces nonterminal differentiation. This fraction was designated CEPH. To prepare CEPH, the pH of citrate anticoagulated human plasma was adjusted to 8.6 using 1 N NaOH. Barium chloride (1 M) was then added to a final concentration of 0.1 M and the pH was readjusted to 8.6 with 1 N NaOH. The suspension was stirred for 45 min at 4°C and sedimented by centrifugation at 4,600 g for 30 min. The barium citrate sediment was resuspended in one-third volume of 0.9% NaCl-0.02 M sodium citrate and mixed for 45 min. This suspension was separated by centrifugation at 4,600 g for 30 min. The supernatant fraction was designated CEPH, i.e., citrate eluate of barium-precipitated plasma at high pH. CEPH was extensively dialyzed against 6.5 mM sodium citrate, pH 7.4, before use.

The preparation of CEPH that is devoid of terminal differentiation-inducing activity is difficult. Although specific lots of CEPH without terminal differentiation activity can be identified, many contain both nonterminal and terminal differentiation-inducing activity. To reduce the terminal differentiation-inducing activity in CEPH, one of two procedures was used. First, medium that contained CEPH was conditioned by culturing in the presence of adipocytes for 4 d followed by sedimentation of the conditioned medium that contained CEPH by centrifugation at 1,000 g for 10 min. Second, CEPH was processed by heparin-agarose affinity chromatography (46). Affi-Gel heparin (Bio-Rad Laboratories, Richmond, CA) was first washed extensively in 10 mM Hepes-6.5 mM Na citrate (pH 7.4); citrate was included to complex calcium ions and thereby inhibits plasma clotting. CEPH, which had been dialyzed against the Hepes-citrate column buffer, was applied to the column at <2:1, vol/vol, CEPH vs. Affi-Gel heparin matrix. Elution with 10 mM Hepes-6.5 mM Na citrate (pH 7.4) was done at a flow rate of 10-20 ml/h. Once all nonheparin binding protein was washed through the column, i.e., the void volume that contains terminal differentiation-inducing activity, the bound protein was eluted with 2.0 M NaCl, 3.0 M urea in 10 mM Hepes-6.5 mM Na citrate (pH 7.4). This material was dialyzed against 6.5 mM Na citrate (pH 7.4). "Processed CEPH" was typically devoid of significant terminal differentiation-inducing activity.

Preparation of Pure Populations of Adipocytes

Cells were induced to undergo nonterminal differentiation by culture in DME that contained fresh or "processed" 25% CEPH, heparin (30 U/ml), and 1 × 10⁻⁵ M biotin (DME/CEPH). Two different tissue culture microenvironments were used to achieve this response. In the first method, cells were grown in DME that contained 10% FCS until they reached ~5 × 10³ cells/cm². The cultures were then fed DME/CEPH twice at 4-d intervals. Purified adipocytes were prepared from these cultures on day 8. In the second method, cells cultured in DME that contained 10% FCS were removed from flasks using 0.1% EDTA in PBS. The dissociated cells were sedimented by centrifugation at 50 g for 5 min, then resuspended in DME/CEPH. The cell suspension was plated onto bacteriological culture plates at ~5 × 10³ cells/cm². Adipocytes were purified from these cultures 5 d thereafter.

To prepare purified adipocytes from the above specimens, the culture media was removed and the adherent cells were gently washed in situ twice in PBS; they were then detached by incubation in 0.1% EDTA in PBS (pH 7.4) for 5 min at room temperature. Adipocytes were preferentially removed by hitting the side of the flask once or twice. The adipocyte suspension was very gently pipetted to dissociate small aggregates of cells, then layered onto a cushion of 10% bovine serum albumin (BSA) in 0.2% EDTA/PBS, pH 7.4, at room temperature. The cells were centrifuged for 10 min at 200 g at 23°C. Cells at the PBS/BSA interface were removed and plated into standard tissue culture flasks in conditioned or processed 10-25% CEPH-containing media. Adipo-

cytes were thereafter allowed to attach for 24 h before use. Such cultures contained 95-100% adipocytes most of which will be shown below to represent nonterminally differentiated cells. Adipocyte differentiation was routinely quantitated by phase microscopy because we and others have established that there is an excellent correlation between such morphological assays and assays of various lipogenic enzyme activities (12, 13, 18). A cell was scored as an adipocyte when the cytoplasm contained numerous refractile lipid droplets.

Proliferative Assays

Two assays were used to quantitate the proliferative potential of purified adipocytes. First, incorporation of [³H]thymidine into trichloroacetic acid (TCA)-precipitable material was used as an assay for DNA synthesis. For these assays purified adipocytes were exposed to [³H]thymidine (1-5 μCi/ml) for 24-48 h in various media, then fixed and processed for autoradiography as previously described (18, 34, 35, 38, 41). Adipocytes with proliferative potential are defined as those cells that show radiolabeled nuclei.

Second, colony formation assays were used to assess the ability of adipocytes to divide and form colonies. For these assays pure populations of adipocytes were plated at low density onto standard tissue culture dishes in conditioned or processed CEPH-containing medium. After 24 h, DME that contained 30% FCS ± 50 μg/ml insulin (some serum lots required added insulin for optimum mitogenic activity) was added to these cultures and the total number of cells that grew in each foci was assayed after 1, 2, or 3 wk. Some such cultures were refed at 4-7-d intervals and some were not. Adipocytes with clonogenic potential are defined as those cells that attach to the plastic substrate and give rise to colonies.

Dedifferentiation Assays

As an additional assay to distinguish cells at the nonterminal and terminal states of differentiation, we evaluated their ability to lose the differentiated phenotype when treated for 3 d with one of the following agents: retinoic acid (RA), 10 μg/ml; 12-O-tetradecanoylphorbol-13-acetate (TPA), 100 ng/ml; methyl isobutyl xanthine (MIX), 5 × 10⁻⁴ M; FCS, 10-30%; tumor growth factor, 30%; epidermal growth factor, 10-100 ng/ml; or dibutyryl cAMP, 1 × 10⁻³ M. In this regard, we previously reported (15) that nonterminally differentiated adipocytes can be induced to lose their differentiated phenotype by some of these reagents, whereas terminally differentiated cells cannot. A cell was considered to have lost the adipocyte phenotype when it contained no or very few small lipid droplets. The biological characteristics of adipocytes and of cells that have been induced to lose the adipocyte phenotype have been described in detail elsewhere (15). The terms "loss of the differentiated phenotype" and "dedifferentiation" are used similarly in this paper because we have recently established that adipocytes that are induced to lose the fat cell phenotype can subsequently be induced to differentiate into another cell type, i.e., into macrophages (42a).

Protein and RNA Synthesis

The effects of chemical inhibitors of protein synthesis were estimated by measurement of the uptake of [³H]methionine into TCA-precipitable material following the method described by Levenson and Housman (21). Briefly, cultures were incubated for 6 h in the presence or absence of cycloheximide (5 μg/ml) or puromycin (10 μM), then cultured in methionine-free DME that contained dialyzed HP, an appropriate dose of inhibitor, and [³H]methionine (5 μCi/ml). Cells were harvested after 1-, 2-, and 3-h intervals by treatment with 0.1 M NaOH for 30 min at 37°C. The protein was precipitated by addition of an equal volume of 10% TCA; BSA (150 μg/ml) was used as carrier. The precipitate was filtered and counted. In the absence of inhibitor the uptake of material into TCA-precipitable material was essentially linear over the time period examined.

RNA synthesis was estimated by measurement of the incorporation of [³H]uridine into TCA-precipitable material in the presence and absence of specific inhibitors, including α-amanitin (10 μg/ml) (45) or cordycepin (5 μg/ml) (22). After appropriate treatment, cultures were fed [³H]uridine (25 μCi/ml) for 3 h. Cultures were then rinsed twice with cold PBS and cells were treated with 1 ml of 10% TCA. Yeast RNA (1 μg/ml) was added as carrier. The precipitate was filtered and counted.

Results

Tissue Culture Model System

The 3T3 T mesenchymal stem cell system is a well-characterized model to study mechanisms for the control of cellular

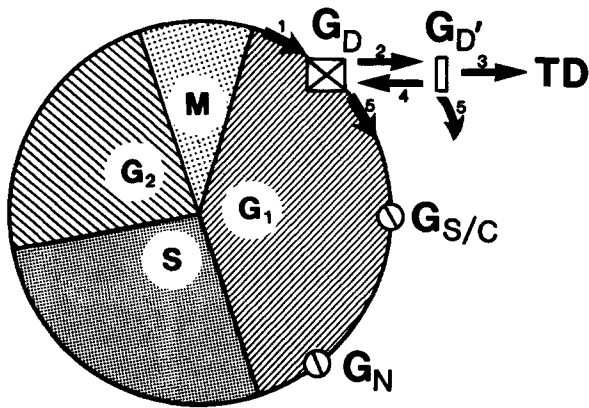


Figure 1. Model for the integrated control of 3T3 T mesenchymal stem cell proliferation and differentiation. It illustrates that before differentiation 3T3 T cells must first growth arrest in the G_1 phase of the cell cycle at a distinct state designated G_D (arrow 1). Cells at G_D can remain quiescent, reinstate proliferation (arrow 5), or undergo differentiation. If differentiation occurs, it involves both nonterminal (arrow 2) and terminal phases (arrow 3). Cells at the nonterminally differentiated state ($G_{D'}$) can remain quiescent, be induced to lose the adipocyte phenotype (arrow 4), reinstate proliferation (arrow 5), or undergo the terminal event in differentiation (arrow 3). The model also illustrates the existence of other restriction points in the G_1 phase of the cell cycle—more specifically, the growth factor-dependent restriction point ($G_{S/C}$) and the nutrient deficiency-induced restriction point (G_N).

differentiation. Fig. 1 presents a schematic model that illustrates the steps involved in control of cellular differentiation and proliferation in this cell type. The model shows that there are three distinct steps involved in adipocyte differentiation in 3T3 T cells. In the first step, cells growth arrest at a distinct predifferentiation state in the G_1 phase of the cell cycle; this state is designated G_D . In the second step, cells express the differentiated phenotype but retain proliferative potential. These cells are defined as being at the nonterminal $G_{D'}$ state of differentiation. In the final step, cells lose proliferation potential and enter the terminal differentiation state designated TD. This paper focuses on the transition from the $G_{D'}$ state to the TD state (18, 34, 35, 38, 41).

Characterization of Purified Adipocytes

Previous studies suggested that adipocytes that develop during culture in heparinized DME that contains CEPH, as above, are primarily nonterminally differentiated (41) because they retain their ability to reinstate proliferation and/or to lose the differentiated phenotype. To quantitatively study the process of terminal differentiation, we developed a gradient method to isolate essentially pure populations of nonterminally differentiated adipocytes. The morphology of these adipocytes is shown in Fig. 2A. In such cultures 95–100% of the cells exhibited adipocyte morphology, i.e., the cells contained multiple large cytoplasmic lipid droplets. It has been shown elsewhere that such cells also exhibit high levels of enzymes associated with lipid metabolism (12, 13, 18).

To determine whether purified adipocytes were indeed nonterminally differentiated and to further characterize such cells, assays were done to evaluate their response to a variety of agents that either induce proliferation or loss of the differentiated phenotype. Fig. 2, B and C illustrates representative

adipocytes that were induced either to proliferate in the presence of 30% FCS \pm 50 μ g/ml insulin or to dedifferentiate in the presence of $1-5 \times 10^{-4}$ M MIX. More specifically, Table I shows that \sim 80% of the purified adipocytes are nonterminally differentiated because they can be induced to lose the adipocyte when treated with MIX and because they can be induced to undergo clonal proliferation. If the kinetics of clonal proliferation are evaluated it is evident that at least 8 d are required to observe a maximum clonogenic response (Fig. 3). In this regard, it is also possible to evaluate the proliferative potential of adipocytes by another assay, that is, via pulse labeling with a mitogen and [3 H]thymidine for 48 h followed by autoradiographic assays. When this assay is used, lower proliferative responses are detected because $>$ 48 h is required for all $G_{D'}$ cells to enter the “S” phase of the cell cycle (Table I). Despite this fact, autoradiographic assays provide a valuable assay to evaluate the relative proliferative potential of different cell populations.

A variety of other agents were tested for their ability to induce proliferation of cells at the $G_{D'}$ state as assayed by the autoradiographic method. The following agents were tested at the indicated dosage and the proliferative response is given in parenthesis: 10 ng/ml epidermal growth factor (35%); 100 ng/ml TPA (17%); 30% tumor growth factor (12%); 10 μ g/ml RA (6%); 10^{-3} M dibutyryl cAMP ($<$ 1%); and 3×10^{-4} M MIX ($<$ 1%). These results represent the mean results of two experiments. Although RA, dbcAMP, and MIX do not induce a significant mitogenic response in $G_{D'}$ cells, they do induce dedifferentiation. In this regard, the following data were obtained. RA (10 μ g/ml) induced 41% dedifferentiation; dibutyryl cAMP (10^{-3} M) induced 76% dedifferentiation; and MIX (3×10^{-4} M) induced 82% dedifferentiation (compare Table I). These data therefore establish that gradient purified adipocytes are nonterminally differentiated.

Induction of the Terminal Event in Adipocyte Differentiation

To determine if purified nonterminally differentiated adipocytes could be induced to undergo the terminal event in differentiation, they were incubated in DME that contained

Table I. Evidence That Gradient Purified Adipocytes Derived from Culture in Medium Containing the Human Plasma Fraction CEPH Are Nonterminally Differentiated

Treatment	Proliferative response		Dedifferentiation response
	Labeled nuclei	Clonogenic potential	Loss of the differentiated phenotype
	%	%	%
None	≤ 1	≤ 1	≤ 1
FCS \pm insulin	35–50	65–80	—
MIX	—	—	75–85

Adipocytes were isolated after culture in DME/CEPH on tissue culture flasks for 8 d. The adipocytes were purified on a BSA gradient and were replated in conditioned DME/CEPH for 24 h. All such cultures were $>$ 95% adipocytes. Thereafter the cells were assayed for their ability to incorporate [3 H]thymidine into DNA or to proliferate in clonogenic assays when cultured in 30% FCS \pm 50 μ g/ml insulin; insulin was added only to certain lots of serum which required it for maximum mitogenic effect. Assays were also done to determine whether such cells could be induced to lose the adipocyte phenotype by treatment with MIX (3×10^{-4} M). The data summarize the results of $>$ 25 experiments. Although some variability was evident in this extensive group of studies, the results always fell within the indicated ranges.

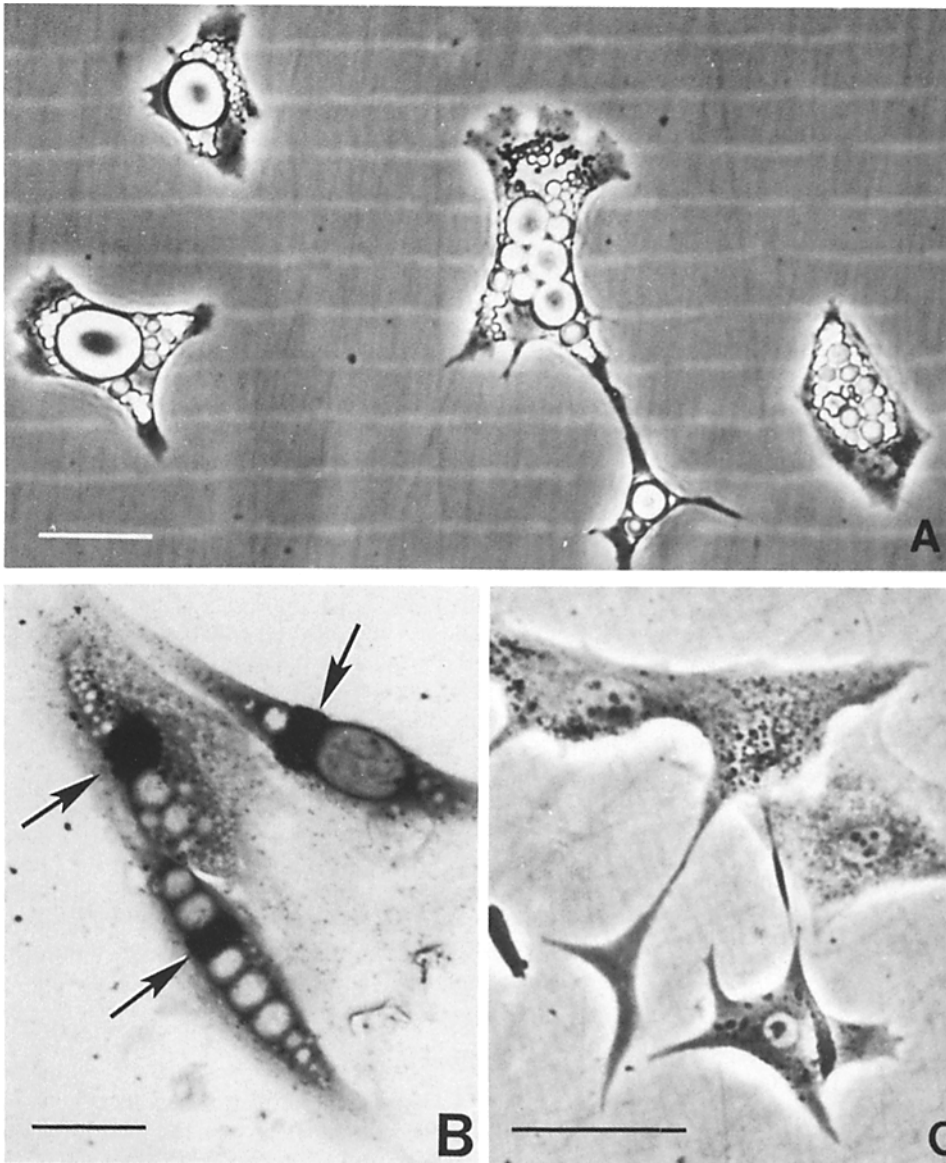


Figure 2. Morphology of purified nonterminally differentiated adipocytes and similar cells induced to proliferate or to lose the differentiated phenotype. (A) Phase micrographs of nonterminally differentiated adipocytes cultured in DME/CEPH and then purified on a BSA gradient. The field shown is representative of all cultures wherein >95% of the cells show large refractile lipid droplets characteristic of the adipocyte morphology. (B) Bright field micrographs of toluidine blue-stained autoradiographs. Purified nonterminally differentiated adipocytes were cultured in 30% FCS \pm 50 $\mu\text{g}/\text{ml}$ insulin and [^3H]thymidine for 48 h, then fixed and processed for autoradiography. Cells that exhibit large lipid droplets also show black-labeled nuclei (arrows). (C) Phase micrographs of purified nonterminally differentiated adipocytes that were exposed to MIX for 72 h to induce loss of the differentiated phenotype. Such cells show decreased numbers and sizes of lipid droplets and assume a flattened morphology characteristic of the undifferentiated cell. Bars, 0.05 mm.

whole HP for various intervals (HP contains terminal differentiation-inducing factors) and they were then assayed for their ability to proliferate or undergo dedifferentiation. Studies were also done to evaluate the characteristics of adipocytes that were initially induced to undergo terminal differentiation in medium that contained whole plasma. The later experiments were technically difficult because such cells did not form a discrete band on BSA gradients and they also showed a very low plating efficiency.

Table II shows that nonterminally differentiated adipocytes maintained in DME/CEPH retain their ability to respond to mitogenic and dedifferentiation-promoting agents, while cells cultured in DME/HP lose responsiveness to these agents. More specifically, cells in DME/CEPH retain the ability to undergo DNA synthesis when treated with serum \pm insulin and they also retain the ability to lose the adipocyte phenotype when treated with MIX. By contrast, cells treated with HP show an 89% reduction in proliferative potential and a 78% reduction in responsiveness to MIX.

The differences in proliferative response between nonter-

minally and terminally differentiated adipocytes detected by 48-h pulse exposure in [^3H]thymidine could reflect differences in the time required for cells at the two stages to enter the cell cycle rather than a qualitative difference in their proliferative potential. This is so because, as discussed above, many adipocytes require >48 h to enter into DNA synthesis after mitogenic stimulation. Clonogenic assays were therefore also done. Purified nonterminally differentiated adipocytes were plated at low density, i.e., 100–500 cells/dish, and allowed to attach to DME/CEPH for 1 d. They were then exposed to DME/CEPH or DME/HP for 12 d and thereafter refed DME/30% FCS \pm 50 $\mu\text{g}/\text{ml}$ insulin for an additional 12 d. Colony number and size was then determined.

The results in Fig. 4 show that ~80% of nonterminally differentiated adipocytes formed colonies when cultured in DME/30% FCS \pm 50 $\mu\text{g}/\text{ml}$ insulin and the vast majority of these consisted of at least 128 cells. In contrast, cells incubated in DME/HP to induce terminal differentiation showed a markedly reduced clonogenic capacity. In fact, >80% of such cells showed no proliferative response and the remainder gave

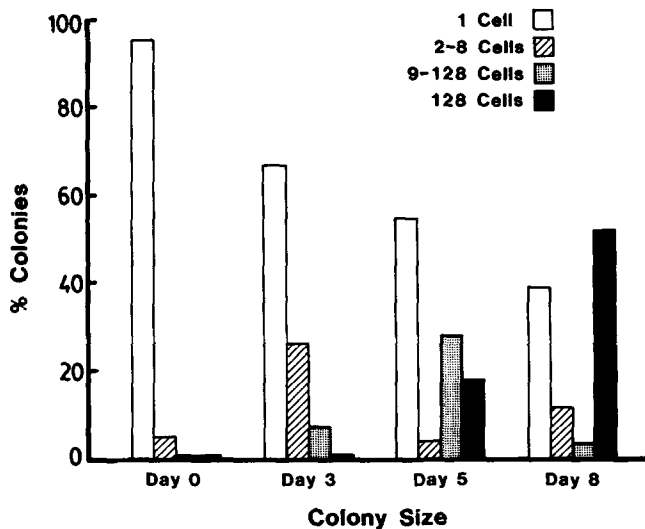


Figure 3. Clonal proliferation assay of nonterminally differentiated adipocytes induced by 30% FCS \pm 50 μ g/ml insulin. Purified adipocytes prepared on BSA gradients (see Materials and Methods) after culture in DME/CEPH for 8 d were plated at low (clonal) densities in conditioned DME/CEPH for 24 h to facilitate attachment. They were then refed 30% FCS \pm 50 μ g/ml insulin. At the indicated times thereafter the number of cells at each colony was counted. The total number of foci remained essentially constant. The figure shows that the majority of purified, differentiated cells reside at the G_D state and not the terminal differentiation state. The data represent the results of a typical experiment; each data point is the mean of duplicate specimens. Comparable results were obtained in more than five experiments.

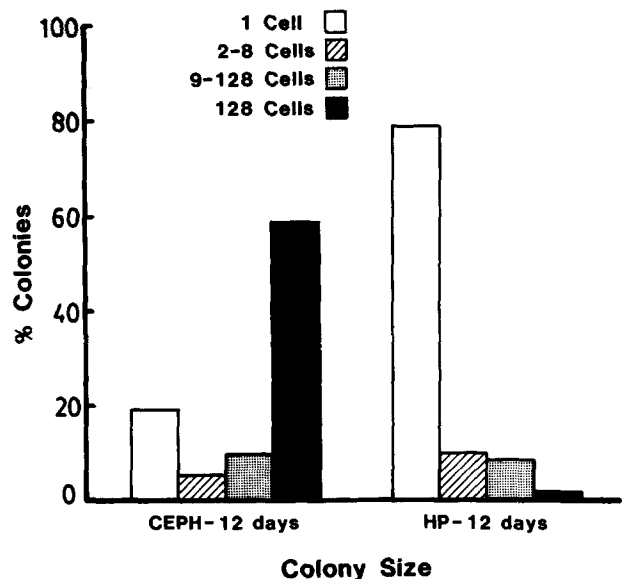


Figure 4. Relative clonogenic potential of nonterminally and terminally differentiated adipocytes. Purified, nonterminally differentiated adipocytes (>98%) were prepared on BSA gradients after culture in DME/CEPH for 8 d. They were then plated at low (clonal) densities in conditioned DME/CEPH for 24 h to facilitate attachment. Thereafter they were refed fresh heparinized DME that contained either 25% CEPH or HP for 12 d. The cultures were next fed DME that contained 30% FCS \pm 50 μ g/ml insulin for an additional 12 d at which time the colonies were counted. In this regard, the total number of colonies per plate remained constant during this interval.

Table II. Induction of the Terminal Event in Differentiation of Nonterminally Differentiated Adipocytes by Whole HP

Specimen designation	Proliferation response		Dedifferentiation response	
	Labeled adipocytes	Terminal differentiation	Loss of phenotype	Terminal differentiation
		(loss of responsiveness)		(loss of responsiveness)
	%	%	%	%
Control cells	36	—	83	—
Cells in DME/CEPH (4 d)	34	5	79	5
Cells in DME/HP (4 d)	4	89	18	78

Purified populations of adipocytes were prepared from cells induced to differentiate by culture in DME/CEPH. The cells were then fed DME/CEPH or DME/HP and incubated therein for 4 d. The proliferative responsiveness of the cells was then determined by stimulation with 30% FCS \pm 50 μ g/ml insulin and [3 H]thymidine for 48 h followed by autoradiography. To measure the ability of these cells to dedifferentiate, MIX (3×10^{-4} M) was added to cultures for 72 h. The morphology of treated and untreated cultures was then assessed. In this assay, terminal differentiation was calculated as the percent loss of responsiveness, i.e., response in control - response in treated/response in control \times 100.

rise to relatively small colonies. These data provide strong evidence that nonterminally differentiated adipocytes can be purified and can subsequently be induced to undergo the terminal event in differentiation. This is true even though nonterminally differentiated and terminally differentiated cells show a comparable morphology (Fig. 5).

Biological Processes Involved in the Terminal Event in Differentiation

To determine whether induction of the terminal event in differentiation is a simple or complex process, nonterminally differentiated adipocytes were refed either DME/CEPH or DME/HP and at sequential times thereafter the cells were tested for their ability to respond to various agents. Fig. 6A shows the proliferative response of cells to 30% FCS \pm insulin. Cells cultured in DME/CEPH for as long as 8 d responded to 30% FCS with DNA synthesis, although the mitogenic response to serum decreased slightly with time. In contrast, cells cultured in DME/HP lose their ability to enter DNA synthesis within 4-6 d.

Dedifferentiation assays were also done to evaluate the complexity of the process of terminal differentiation. Fig. 6B shows the effect of MIX. Cells maintained in DME/CEPH retain their ability to dedifferentiate when treated with MIX, whereas essentially all cells cultured in DME/HP lost this response within 10-14 d. Fig. 6C, however, shows a distinctly different result in that adipocytes maintained in either DME/CEPH or DME/HP both lose responsiveness to RNA treatment over an 8-10-d interval. Similar results were obtained with two- to fivefold higher concentrations of RA.

Finally, the data in Fig. 6D show an even more complex response when cells cultured in DME/CEPH and DME/HP were treated with TPA. Purified nonterminally differentiated cells initially showed minimal responsiveness to TPA; however, cells maintained in DME/CEPH gradually became more responsive to TPA and on day 9, >60% of the cells could be induced to lose the adipocyte phenotype in response to TPA.

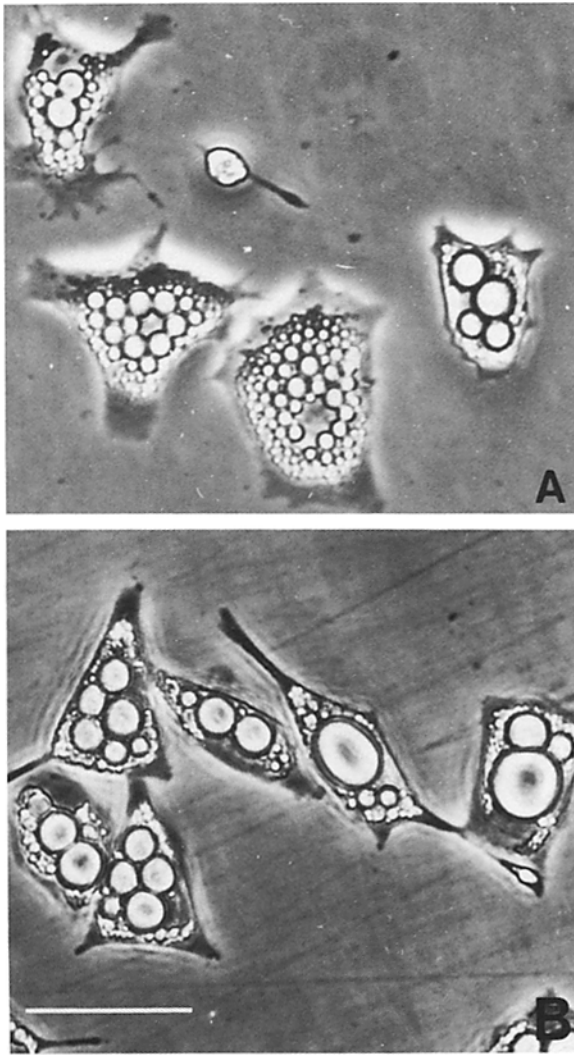


Figure 5. Phase micrographs of nonterminally and terminally differentiated cells. (A) Nonterminally differentiated, purified adipocytes were cultured in DME/CEPH for 8 d before analysis. Such cells exhibit multiple intracellular lipid droplets. (B) Terminally differentiated, purified adipocytes were fed DME/HP for 8 d before analysis, and these cells also exhibit large lipid droplets. Although terminally differentiated adipocytes, such as those illustrated in Fig. 5B, usually contain larger lipid droplets than nonterminally differentiated cells, this is not an absolute characteristic. In some experiments both G_D and terminal differentiation cells show a "signet ring" appearance. Bar, 0.05 mm.

Adipocytes cultured in DME/HP also initially lost the adipocyte phenotype when exposed to TPA; however, continued culture in DME/HP for longer periods (6–9 d) resulted in loss of TPA responsiveness.

In summary, immediately after their isolation, nonterminally differentiated adipocytes are responsive to FCS \pm insulin, RA, and MIX, but are relatively unresponsive to TPA treatment. Nonterminally differentiated adipocytes cultured in DME/CEPH for longer intervals retain responsiveness to treatment with FCS \pm insulin and MIX, gain responsiveness to treatment with TPA, and lose responsiveness to treatment with RA. By contrast, cells cultured in HP lose the ability to respond to all of these agents over a 4–6 d period. These

experiments suggest that transition from a nonterminal to terminal state of differentiation involves at least two metabolic steps as illustrated in Fig. 7.

Effects of a Pulse Exposure of Nonterminally Differentiated Cells to Human Plasma-containing Medium

The data presented in Fig. 6 and our previous studies (18, 41) suggest that the terminal event in differentiation requires an interval of 4–6 d to be fully expressed. The following experiments were done to determine whether the continued presence of HP was required for induction of the terminal event in differentiation or whether the process could be initiated after a brief exposure to HP. For this study, cultures of purified nonterminally differentiated adipocytes were exposed to DME/HP for varying lengths of time, the HP was then removed by rinsing the cultures repeatedly in PBS, and the cells were then cultured for an additional 4 d in DME/CEPH. Such cells were then assayed to determine the interval of exposure to an inducer that is required for transition from a nonterminal to terminal state of differentiation. Terminal differentiation in these assays was measured by loss of mitogenic responsiveness to 30% FCS \pm insulin and by loss of MIX-induced dedifferentiation responses.

Fig. 8 presents the results of these experiments. Exposure of nonterminally differentiated adipocytes to DME/HP for 1–6 h induced ~50% of the cells to undergo the terminal event in differentiation 4 d thereafter. Exposure of nonterminally differentiated adipocytes to HP-containing medium for 12–24 h caused ~75% of the cells to undergo the terminal event in differentiation 3 d thereafter. Finally, if nonterminally differentiated cells were exposed to HP-containing medium for ≥ 48 h, maximum terminal differentiation was observed 2 d later. Therefore, although transition from a nonterminal to a terminal state of differentiation requires ~4 d to be completely expressed, a 12–24-h pulse exposure to HP-containing medium is sufficient to trigger the terminal event in the differentiation of many of the cells. Additional dose-response experiments (not shown) established that the concentration of HP in the medium that is required to induce the terminal event in differentiation is $\geq 5\%$; therefore residual HP that might be bound to cells probably does not account for the induction of the terminal event in differentiation in pulse-treated cells.

Effects of Metabolic Inhibitors on the Induction of the Terminal Event in Differentiation

To determine whether specific metabolic inhibitors modulate expression of the terminal event in differentiation, purified nonterminally differentiated adipocytes were exposed to DME/HP in the presence or absence of drugs for a 24-h interval after which time the cultures were rinsed extensively and refed DME/CEPH. 4 d thereafter the cells were assayed to determine if the terminal event in differentiation had occurred. These assays measured the cells' responses to MIX or 30% FCS \pm insulin treatment as described above. As designed, these studies specifically assayed whether protein or RNA synthesis are required during the initiation phase of terminal differentiation, i.e., the first 24 h. This experimental design represented the only feasible approach using most metabolic inhibitors because longer exposure of cells to these

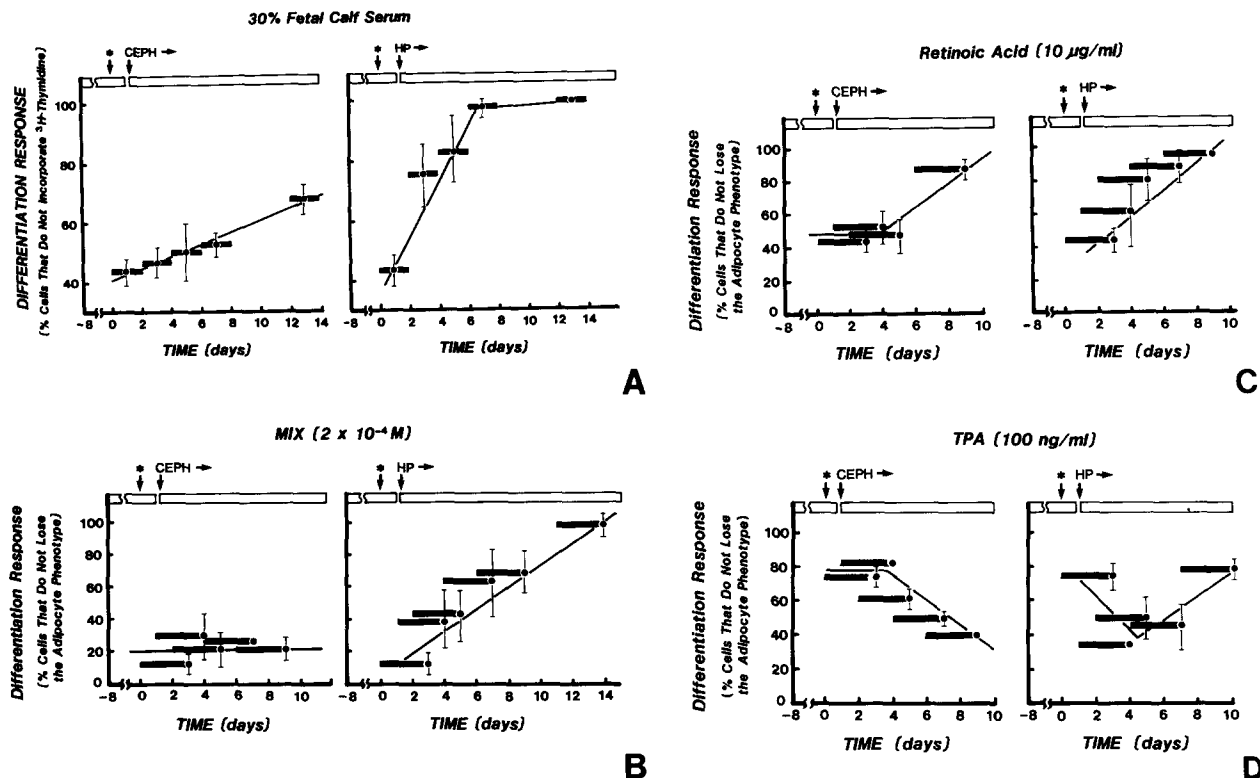


Figure 6. Biological processes associated with the terminal event in differentiation. For all experiments, nonterminally differentiated adipocytes were purified from cultures incubated with DME/CEPH for 8 d at the time indicated by the asterisk (*). The purified adipocytes were then replated in conditioned DME/CEPH to facilitate attachment. After 24 h, the cultures were then fed heparinized DME that contained either CEPH or HP at the time indicated by the arrows. At various times thereafter cultures were exposed to different agents for the periods indicated by the solid bars. The cultures were assessed either autoradiographically in proliferation assays or morphologically for loss of differentiation assays at the end of the period. Data are presented as differentiation response as explained in A–D below. (A) Response of purified adipocytes cultured in medium that contained CEPH or HP, then subsequently treated with 30% FCS \pm 50 μ g/ml insulin. The data were derived from autoradiographic assays of thymidine incorporation. The differentiation response is expressed as the percentage of cells that did not incorporate [3 H]thymidine. (B, C, and D) Response of purified adipocytes cultured in medium that contained CEPH or HP, then treated with 3×10^{-4} M MIX (B), 10 μ M RA (C), and 100 ng/ml TPA (D) for 72 h. The data were derived from morphological analysis of the percent adipocytes before and after treatment. The differentiation response is expressed as the percent of cells that did not lose the adipocyte phenotype.

agents resulted in significant cytotoxicity. The results of these studies are presented in Table III.

The effects of the protein synthesis inhibitors, cycloheximide and puromycin, on induction of the terminal event in differentiation were examined first (Table III). Both drugs inhibited induction of the terminal event in differentiation at concentrations that also inhibited 70–80% of protein synthesis as determined by measurement of the uptake of [35 S]methionine into TCA-precipitable material. More specifically, these two protein synthesis inhibitors blocked induction of the terminal event in differentiation by 70–87%.

Similar studies were next done with α -amanitin, which inhibits ~70% of the activity of RNA polymerase I (45), and cordycepin, which inhibits polyadenylation of mRNA (22). The data in Table III show that neither cordycepin nor α -amanitin inhibited induction of the terminal event in differentiation. In this regard, it was demonstrated that neither of these drugs was cytotoxic.

Discussion

Nonterminal and terminal states of differentiation have been established to exist in 3T3 T adipocytes. In this paper, we report the development of methods to isolate purified popu-

lations of nonterminally differentiated cells and show that these cells can be maintained at this nonterminal state of differentiation for longer than 1 wk. We also demonstrate that nonterminally differentiated cells can be induced to undergo the terminal event in differentiation when they are incubated in medium that contains whole HP. Transition between these two states is shown to involve a complex metabolic process that consists of at least two substrates that can be distinguished by the demonstration of differential responsiveness to agents that include RA, TPA, and MIX.

Induction of the terminal event in differentiation is shown to be triggered by short-term exposure of nonterminally differentiated cells to HP even though the terminally differentiated phenotype was not fully expressed for 4 d thereafter. Finally, induction of the terminal event in differentiation is shown to be blocked by drugs that inhibit protein synthesis but that it is unaffected by two drugs that affect mRNA synthesis or processing. These results suggest that synthesis of specific proteins may be associated with the induction of the terminal event in differentiation. In this regard, results to be published elsewhere establish that modulation in the expression of only eight major polypeptides occurs during the transition from the nonterminal to the terminal state of adipocyte differentiation.

These observations clearly demonstrate that in the 3T3 T mesenchymal stem cell system, expression of the differentiated phenotype and loss of proliferative potential are separately controlled processes and that expression of the differentiated phenotype is not sufficient to induce loss of proliferative potential. That is, a separate and specific control process must be required to induce the irreversible loss of proliferative potential that occurs in association with the induction of the terminal event in differentiation. We have identified and purified a 20,000-fold human plasma protein that regulates the terminal event in differentiation and we have designated this protein as a proliferin (Wier, M. L., and R. E. Scott, manuscript submitted for publication).

These observations require that previously proposed concepts for the control of terminal cellular differentiation be modified. For example, it has been suggested that a single early event in the differentiation process programs cells to subsequently lose proliferative potential in association with

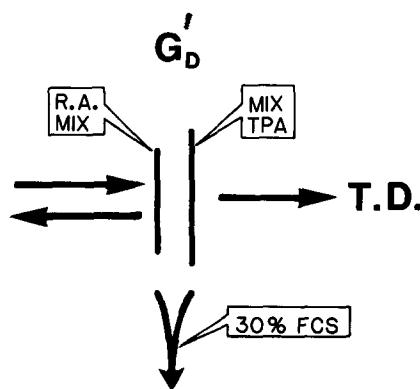


Figure 7. Model to illustrate the two biological steps involved in the transition from the nonterminal to terminal state of differentiation. This model represents a focal expansion of the model presented in Fig. 1 specifically involving the later stages in the $G_D/G_D'/TD$ complex. It shows that cells at the first substage of G_D' can be induced to lose the differentiated phenotype when treated with RA and MIX but not with TPA. By contrast, cells at the second substage of G_D' can be induced to lose the differentiated phenotype when treated with MIX and TPA but not with RA. Cells at both substrates are at G_D' and nonterminally differentiated because they can both be induced to undergo proliferative responses when treated with FCS \pm insulin. By contrast, cells at the terminally differentiated state are not responsive to any of these agents.

differentiation (9, 14). Contrary to this proposal, our data show that nonterminally differentiated adipocytes retain their ability to lose the differentiated phenotype and to reinitiate proliferation for extended intervals. In fact, we showed that $\sim 80\%$ of such cells can undergo clonal proliferation. In this regard, we have also recently reported the isolation of clones of 3T3 T mesenchymal stem cells that are selectively defective in their ability to undergo the terminal event in differentiated (47). Other theories of terminal differentiation have also suggested that loss of proliferative potential results from an inhibitory effect of differentiation gene products on DNA

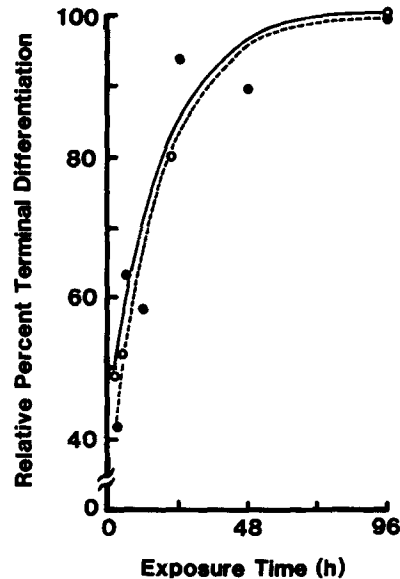


Figure 8. Kinetics of the induction of the terminal event in adipocyte differentiation. Purified nonterminally differentiated adipocytes were incubated in medium that contained HP for 1, 3, 6, 12, 24, 48, or 96 h. They were then rinsed and recultured in DME/CEPH, a medium that does not induce terminal differentiation for an additional interval until the total duration of the experiment was 96 h. The cultures were then treated with 3×10^{-4} M MIX (O) or were fed medium that contained 30% FCS \pm 50 $\mu\text{g/ml}$ insulin (●) as described previously. The data are presented as the relative percent terminal differentiation wherein the absolute extent of terminal differentiation observed in cells incubated in DME/HP for 96 h or longer was defined as 100% response. The data show that pulse exposure of nonterminally differentiated adipocytes to HP for 24 h was adequate to induce a near maximum terminal differentiation response.

Table III. Effect of Metabolic Inhibitors on Induction of the Terminal Event in Differentiation

Differentiation-promoting culture medium	Drug	Inhibition of precursor uptake		Proliferation response		Dedifferentiation response	
		[^3H]Methionine	[^3H]Uridine	Labeled nuclei	Inhibition of terminal differentiation	Loss of differentiated phenotype	Inhibition of terminal differentiation
		%	%	%	%	%	%
DME/CEPH	None	—	—	36	—	85	—
DME/HP	None	—	—	6	—	18	—
DME/HP	Cycloheximide	68	—	27	70	76	87
DME/HP	Puromycin	82	—	27	70	73	82
DME/HP	α -Amanitin	—	66	6	0	—	—
DME/HP	Cordycepin	—	—	0	0	—	—

The experimental procedures for cell preparation and to assay proliferation (30% FCS \pm 50 $\mu\text{g/ml}$ insulin) and differentiation (3×10^{-4} M MIX) responses were described previously. The concentrations of the drugs that were used are: cycloheximide, 5 $\mu\text{g/ml}$; puromycin, 1 mM; α -amanitin, 10 $\mu\text{g/ml}$; and cordycepin, 5 $\mu\text{g/ml}$.

synthesis, cellular proliferation, and cell division (5, 19, 30). In the 3T3 T mesenchymal stem cell system this cannot hold true because a stable nonterminally differentiated state clearly exists and it can be maintained.

The possibility that our results on 3T3 T mesenchymal stem cells are not representative of other cell types can also easily be dismissed. Although it is true that 3T3 T mesenchymal stem cells are not completely normal (18, 34, 35, 38, 41) and may not express all normal differentiation control mechanisms, there is abundant additional evidence from both in vivo and in vitro studies for the existence of a comparable nonterminal state of differentiation in many cell types and their regulated transition to a state of terminal differentiation. For example, in vitro studies on myoblasts have shown that even after they have withdrawn from the cell cycle and have begun to express myosin they can be induced to reenter the cell cycle when exposed to growth media (7). A reversible differentiated state has also been identified in mast cells (53), melanoma cells (1), erythroleukemia cells exposed to hemin (4, 6), neuroblastoma cells exposed to butyrate (10), and human epithelial cells (20, 48, 50). Furthermore, variants of erythroleukemia (24) and rat myoblast (26) cell lines have been identified which differentiate but do not lose proliferative potential under selected culture conditions. These results suggest that the G_D -like state may be an important phase in the in vitro differentiation of many cell types.

In vivo studies on cardiac myocytes and neuroblasts during development also suggest that many cells that are terminally differentiated in an adult animal pass through a nonterminal phase during development (28, 31). Finally, in vivo studies of lymphocytes and liver, Schwann, and smooth muscle cells suggest that cells can be maintained in a nonterminally differentiated state for extended periods even in adult tissues before terminal differentiation (2, 17, 23, 33).

While all such observations have provided evidence for the existence of a nonterminally differentiated state and the transition of such cells to a subsequent terminal state of differentiation, the 3T3 T mesenchymal stem cell system is the only one in which it has been possible to rigorously control and study the transition from the nonterminal to terminal states of differentiation. Most important, this model provides an excellent system to characterize physiological proteins and other agents that may induce the terminal event in differentiation and to characterize the metabolic processes that occur during the terminal event in cellular differentiation.

Characterization of the biological and molecular mechanisms that irreversibly restrict a cell's proliferative potential is also of potentially great biological and pathological significance especially as it relates to the mechanisms of carcinogenesis. Although the overwhelming majority of publications concerning the pathobiology of carcinogenesis have focused on analysis of the role of growth factors and oncogenes that are associated with activation of proliferative responses, it is equally important to consider the possibility that carcinogenesis results in significant part from the expression of defects in regulatory mechanisms that suppress aberrant cellular proliferation. That is, an early stage in carcinogenesis could be associated with loss of proliferation suppressor mechanisms associated with the terminal event in differentiation rather than with the activation of positive regulators of proliferation. The results of our previous studies (36, 37, 39, 40, 47) and

those of other investigators (32, 42, 43, 52) support this possibility.

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