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An initiator of carcinogenesis selectively and stably inhibits stem cell differentiation: A concept that initiation of carcinogenesis involves multiple phases

(differentiation and proliferation control/ultraviolet irradiation/cell cycle/proadipocyte stem cells)

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ABSTRACT **A concept of carcinogenesis was recently devised in our laboratory that suggests the development of defects in the control of cell differentiation is associated with an early phase of carcinogenesis. To test this proposal directly, the effects of an initiator of carcinogenesis (i.e., UV irradiation) on proadipocyte stem cell differentiation and proliferation was assayed. In this regard, 3T3 T proadipocytes represent a nontransformed mesenchymal stem cell line that possesses the ability to regulate its differentiation at a distinct state in the G_1 phase of the cell cycle as well as the ability to regulate its proliferation at two additional G_1 states that are induced by culture in growth factor-deficient or nutrient-deficient microenvironments. The results establish that a low dosage of 254 nm UV irradiation selectively and stably inhibits the differentiation of a high percentage of proadipocyte stem cells without significantly altering their ability to regulate cellular proliferation in growth factor-deficient or nutrient-deficient culture conditions. Differentiation-defective proadipocyte stem cells are demonstrated not to be completely transformed but to show an increased spontaneous transformation rate, as evidenced by the formation of type III foci in high density cell cultures. These data support the role of defects in the control of differentiation in the initiation of carcinogenesis. Other data, however, also suggest that additional cellular defects must be expressed for a cell to be completely initiated. These observations support a concept that the initiation of carcinogenesis involves multiple phases.**

Initiation is the early phase of carcinogenesis; however, the biological characteristics that distinguish initiated cells have not been definitively established (1–5). Clinical studies suggest that between 15 and 30 years are required for an initiated cell to develop into an invasive cancer (6). Clinical observations have also shown that many cancers develop from preneoplastic cells that differentiate aberrantly. These include cells of the preleukemic diseases and cells of metaplastic and dysplastic lesions of epithelial tissues (7, 8). These clinical observations and the results of our previous experimental studies (9–11) and those of others (12–14) form the data base for our concept of carcinogenesis. This concept proposes that the development of defects in the control of differentiation represents an early phase of carcinogenesis, whereas the development of defects in the control of proliferation represents a later phase in the transformation process. In this regard, we have suggested that the development of defects in the control of differentiation and proliferation is mediated at distinct cell cycle states (Fig. 1). This suggestion is based on data that demonstrated that, in proadipocyte stem cells, cellular differentiation is mediated at a distinct complex G_1 arrest state, designated $G_D/G_{D'}/TD$ (15–17), and that cellular proliferation is further mediated at other G_1 arrest states, such as those induced by growth factor deficiency, designated $G_{S/C}$, or by nutrient deficiency, designated G_N (Fig. 1) (9, 10, 15, 18).

To test the concept that an early phase of carcinogenesis is associated with the expression of defects in the control of differentiation, we performed the experiments reported in this paper. In these studies, we used incompletely initiated (19) and nontransformed proadipocyte stem cells (11). The results show that UV irradiation, an initiator of carcinogenesis (20, 21), selectively and stably inhibits proadipocyte stem cell differentiation without abrogating other mechanisms for the control of proliferation, such as those mediated at $G_{S/C}$ and G_N. The results also show that the development of defects in the control of differentiation occurs in a high percentage of UV-irradiated cells but that the vast majority of such cells are not transformed even though they do show an increased rate of spontaneous transformation.

These observations have been synthesized with other data into an expanded concept that suggests that multiple phases are involved in the initiation of carcinogenesis. These include the development of (*i*) genetic instability and defects in the control of cellular commitment to differentiation, (*ii*) defects in the expression of the differentiated phenotype, and (*iii*) acquisition of responsiveness to tumor-promoting agents.

METHODS

Cell Cultures and UV Irradiation. The proadipocyte stem cells used in this study are designated 3T3 T and were supplied by L. Diamond (22). These cells were cultured as described (15–17) and were repeatedly found to be free of mycoplasma contamination (23). They were passaged at 1–3 $\times 10^3$ cells per cm^2 and were allowed to grow in Dulbecco's modified Eagle's medium (DME medium)/10% fetal calf serum for 24–48 hr prior to UV irradiation. UV irradiation was with a GE 15T8 lamp that supplied predominantly 254 nm irradiation. This UV source was calibrated with a Stuart 3 thermopile with secondary calibration against a Hilger-Watts FT17.1 thermopile with output monitored by a Keithley 150B microvoltmeter in the laboratory of M. J. Peak (Argonne National Laboratory, Argonne, IL). Cultures were irradiated at 0–270 erg/mm^2 in either single or split dosage after transiently removing the tissue culture medium. Thereafter, the cells were cultured for ≥24 hr prior to use after the extent of UV-induced cytotoxicity was determined (24, 25).

Induction of Differentiation. Proadipocyte differentiation was induced by incubating low density cultures (≈3 $\times 10^3$ cells per cm^2) in heparinized (30 units/ml) DME medium/25% human plasma as described (15–17, 26). Twelve to 16 days thereafter, the percentage of cells with an adipocyte morphology was quantitated by phase microscopy (15–17). This assay correlates well with enzymatic assays of differen-

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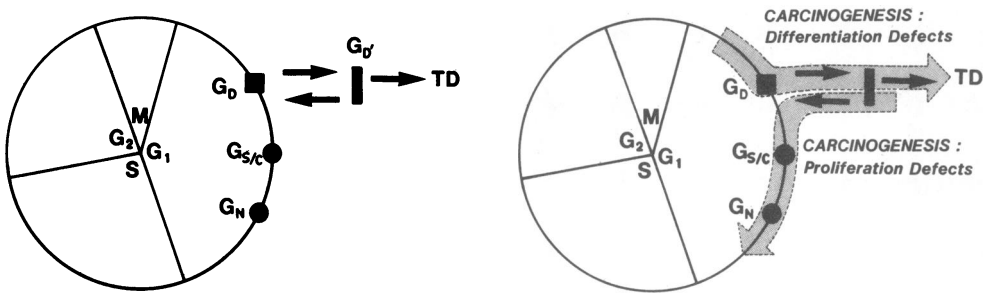

FIG. 1. Concept for the role of expression of cell cycle-dependent defects in the control of cellular differentiation and proliferation in carcinogenesis. Cell cycle model (*Left*) illustrates the three major growth arrest states that exist in the G_1 phase of the cell cycle in proadipocyte stem cells. G_D represents the predifferentiation arrest state at which cells can integrate the control of both proliferation and differentiation. Cells at G_D can either reinitiate proliferation or they can differentiate. If differentiation occurs, it proceeds through at least two steps. Cells at G_D first undergo nonterminal differentiation and then arrest at the state designated $G_{D'}$. At this state, the integrated control of proliferation and differentiation can also be mediated, because such cells can either terminally differentiate (i.e., TD) or they can reinitiate proliferation after loss of the differentiated phenotype and return to G_D. Cells at $G_{D'}$ can also reinitiate proliferation without losing the differentiated phenotype (not illustrated); that is, they can proliferate while retaining the adipocyte phenotype. The second state at which G_1 growth arrest can be induced results from depletion of growth factors in the medium. This can result from so-called contact inhibition, or it can be induced by culture of low density cells in serum-depleted medium. This arrest state is designated $G_{S/C}$. The third state at which growth arrest can be induced results when proadipocyte stem cells are cultured in nutrient-deficient medium. This arrest state is designated G_N. Our concept of carcinogenesis related to this model is presented (*Right*). It suggests that an early phase in carcinogenesis is associated with the development of defects in the control of cell differentiation (upper dotted arrow) and that a later step in carcinogenesis results from the development of aberrant growth control properties, which allows such cells to override both primary and backup growth regulatory mechanisms, such as those mediated at $G_{C/S}$ and G_N (lower dotted arrow).

tiation (15–17) and detects both nonterminally and terminally differentiated cells. To distinguish these two states of differentiation, adipocytes were also incubated in DME medium/30% fetal calf serum with or without ± 50 μg of insulin per ml and [^{3}H]thymidine (3–5 μCi/ml; 1 Ci = 37 GBq) for 48–72 hr, and the nonterminally differentiated cells that retained proliferative capacity were thereafter determined by autoradiographic analysis of random counts of ≈500 cells per specimen.

Induction of Growth Arrest by Serum or Nutrient Deficiency. Murine embryo cells, such as 3T3 T, can undergo growth arrest in the G_1 phase of the cell cycle as a result of growth factor or nutrient deficiency (18, 27). Growth factor-dependent arrest resulting from high density/contact (G_C) or from serum deprivation at low density (G_S) was induced as described (15). Nutrient-dependent arrest (G_N) resulting from isoleucine deprivation at low density was also induced as described (15), the only modification being that low density cultures were plated at 3×10^3 cells per cm^2. Cell cycle analysis was done with a FACS IV flow microfluorimeter and cell cycle distributions were studied as described (9, 15, 28).

Cell Transformation. Two *in vitro* methods were used to detect the transformed phenotype. These included the focus-formation assay (21) and assay of anchorage-independent growth in soft agar (11). Both assays were carried out by using published procedures (11, 21).

RESULTS

Effects of UV Irradiation on Differentiation. Exposure of 3T3 T proadipocytes to UV irradiation (254 nm) was first assayed to determine its effects on adipocyte differentiation. Exponentially growing low density cells were UV irradiated in a single dosage of 10, 20, 40, 60, or 90 erg/mm^2 and thereafter cultured for 48–72 hr. They were then repeatedly refed differentiation-promoting medium (i.e., heparinized DME medium containing 25% human plasma), and the extent of adipocyte differentiation was quantitated for 16 days. The results showed that UV irradiation inhibited differentiation in a dose-dependent manner: 10–20 erg/mm^2 caused no significant effect, UV dosages of 40–60 erg/mm^2 had an intermediate effect, and dosages of UV irradiation of ≥90 erg/mm^2 caused maximum inhibition of differentiation.

To substantiate that this effect was a reproducible phenomenon, a series of additional assays were performed over a 1.5-year period. These data are summarized in Fig. 2; they show that single or cumulative dosages of UV irradiation of 40–90 erg/mm^2 reproducibly inhibited differentiation. The inhibition induced by these treatments showed a highly significant difference compared to cells that received either no UV irradiation or dosages ≤30 erg/mm^2. For example, 40 erg/mm^2 inhibited differentiation 40%–60% and 60 and 90 erg/mm^2 inhibited differentiation 50%–75%. After these studies, three UV irradiated cell lines were established. The cell line designated UV-I was initially treated with UV irradiation at 90 erg/mm^2 in a single dosage. UV-II was initiated after two exposures to UV irradiation at 90 erg/mm^2 at a 7-day interval, and UV-III was initiated after three exposures to UV irradiation at 90 erg/mm^2 at 5- to 7-day intervals.

Since the differentiation of 3T3 T proadipocyte stem cells involves both nonterminal and terminal phases (Fig. 1) and since morphological analysis cannot accurately predict

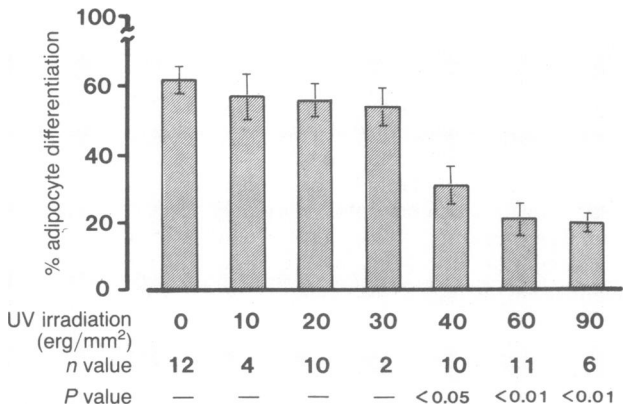

FIG. 2. Summary of effects of UV irradiation on proadipocyte stem cell differentiation in multiple experiments. The data are expressed as mean values ± SEM. The number of experiments performed at each dosage schedule is indicated as *n* value. The results show that dosages of UV irradiation ≥40 erg/mm^2 inhibited differentiation >50%. The *P* value of statistical significance was determined by the Student's *t* test.

which cells express only defects in the control of terminal differentiation, another series of studies was performed. These studies were designed to determine whether UV irradiation induces specific defects in the ability of adipocytes to undergo terminal differentiation. To accomplish this goal, native proadipocyte stem cells and UV-I, UV-II, and UV-III cell lines were maintained in terminal differentiation-inducing medium—i.e., heparinized DME medium/25% human plasma, for 17 days; they were then refed DME medium containing 30% fetal calf serum with or without 50 μg of insulin per ml and [^{3}H]thymidine to induce proliferation. The percentage of adipocytes that were capable of undergoing DNA synthesis was then assayed autoradiographically. The results show that whereas >85% of native cells underwent terminal differentiation, terminal differentiation was significantly reduced in the cells of UV-irradiated lines I, II, and III. That is, of the adipocytes that developed in UV-irradiated cell lines I, II, and III (which were reduced ≈60% compared to native cells), only 53%, 69%, and 50%, respectively, underwent terminal differentiation; the remainder maintained a nonterminal phenotype. This represents a 33% decrease in terminal differentiation potential of the adipocytes that developed in the irradiated cell lines. These data, together with those presented in Fig. 2, demonstrated that UV irradiation inhibits combined nonterminal and terminal differentiation by ≥70%.

Cytotoxic Effect of UV Irradiation. The exposure of cells in culture to UV irradiation causes significant cytotoxicity in a variety of cell types, and in many cases there is a close correlation between the dosages of UV irradiation that cause cytotoxicity and other biological effects (21, 24, 25). We therefore performed three dose–response assays to characterize the cytotoxic effect of UV irradiation on exponentially growing proadipocyte stem cells. The results showed that most cell death occurs within the first 48 hr after exposure to UV irradiation and that thereafter the surviving cells reinitiated exponential growth. Dose–response analysis of the cytotoxic effects of UV irradiation on proadipocyte stem cells showed that it was essentially linear for dosages of UV irradiation of 20–60 erg/mm^2; 20 erg/mm^2 killed 10%–15% of the cells, 40 erg/mm^2 killed 40%–50% of the cells, and 60 erg/mm^2 killed 55%–65% of the cells. Dosages of irradiation of >60 erg/mm^2 however induced a variable increase in cytotoxicity. The dosages of UV irradiation that induced cytotoxic effects were therefore similar to those that inhibited differentiation.

Two interpretations could explain these data. Either UV irradiation could inhibit cellular differentiation and cause cytotoxicity by different mechanisms but with similar dose responses, or both effects could be caused by a common process that involves the selection of differentiation-defective UV-resistant clones. To establish that selection of UV-resistant clones could not explain our results, three separate experiments were performed and all of them gave comparable results. More specifically, if exponentially growing proadipocyte stem cells were initially exposed to UV irradiation at 90 erg/mm^2, 50%–60% of the cells died within 48–72 hr. The residual cells thereafter reinitiated proliferation and grew well and could be passaged. If such cells were then exposed to a second treatment with UV irradiation at 90 erg/mm^2, 50%–60% of the cells were again killed. If this procedure was repeated a third time on the surviving cells, a cytotoxic response of 45% was observed. Therefore, exposure of proadipocyte stem cells to low dosages of UV irradiation did not result in the selection of UV-resistant clones that cannot differentiate, because all such cultures retain a similar cytotoxic response to a fixed dosage of UV irradiation and because all resulting UV-irradiated cell populations showed similar biological characteristics (see below). This conclusion is comparable to that derived from studies on other cell systems (24, 25) and, therefore, suggests that UV irradiation does indeed have specific effects that inhibit both nonterminal and terminal differentiation.

Stability of the UV-Induced Effect on Adipocyte Differentiation. To determine whether the UV-induced effect on the differentiation of proadipocyte stem cells was stable, the three UV-irradiated lines of proadipocyte stem cells described above were used. After irradiation, these cell lines were maintained and passaged without further irradiation, and they were then tested for their ability to differentiate relative to native cells. Differentiation assays were performed periodically for 247 days or ≈8 months. The results presented in Table 1 establish that the UV-induced defects in the ability of proadipocyte stem cells to differentiate were stable because UV-irradiated cell lines showed a consistent and stable inhibition in differentiation potential of ≈60%. (In these experiments, specific terminal differentiation assays were not performed.)

Analysis of the Growth Regulatory Characteristics of UV-Irradiated Proadipocyte Stem Cells. The hypothesis that formed the basis for these studies was that an early stage of carcinogenesis is associated with the development of specific defects in the control of cellular differentiation. A corollary to that hypothesis was the proposal that initiated cells should not show significant defects in their ability to undergo growth regulation at other G_1 states, such as those induced by growth factor deficiency at high cell density (G_C) or low cell densities (G_S), or those induced by nutrient deficiency (G_N).

UV-irradiated proadipocyte cell lines, designated UV-I, UV-II, and UV-III, were therefore analyzed for their ability to growth arrest at these various states in G_1 as well as for their ability to differentiate. Table 2 presents the results of these studies. The data show that relative to untreated 3T3 T proadipocyte stem cells, the UV-irradiated cell lines retain their ability to growth arrest at the G_C, G_S, and G_N states. This was verified not only by their ability to growth arrest at an appropriate saturation density under specific culture conditions, but also by flow microfluorimetric analysis, which showed that when untreated and UV-irradiated cultures were growth arrested all showed >80% of the cells in the G_1 phase of the cell cycle with a $2n$ DNA content (data not shown). Notwithstanding the fact that these cells can regulate their growth at the $G_{S/C}$ and G_N states, all three UV-irradiated cell lines show a marked deficiency in their ability to differentiate (Table 1).

These observations establish that UV irradiation can induce specific stable defects in the ability of 3T3 T proadipocyte stem cells to undergo nonterminal and terminal differentiation without inducing defects in other growth regulatory processes.

Effect of UV Irradiation on Expression of the Transformed Phenotype. It has been previously reported that UV irradiation primarily initiates carcinogenesis (20, 21). However, it

Table 1. Stability of effect of UV irradiation on proadipocyte stem cell differentiation

	Days after initial irradiation								
	2	9	13	18	84	96	118	247	Mean
UV-I	57	—	—	—	45	53	68	—	56
UV-II	—	68	—	61	43	—	77	45	59
UV-III	—	—	65	60	44	—	55	68	58

UV-irradiated proadipocyte cell lines designated UV-I, UV-II, and UV-III were established as described in the text. Thereafter, they were periodically tested for their ability to differentiate in heparinized DME medium containing 25% human plasma relative to untreated specimens. Data are expressed as the percent inhibition of differentiation in UV-irradiated 3T3 T cells relative to untreated 3T3 T cells.

Table 2. Biological characteristics of 3T3 T and UV-irradiated 3T3 T proadipocyte stem cells

Cell phenotype	Differentiation*	G_C arrest†	G_S arrest†	G_N arrest†
3T3 T	86 ± 5	22 ± 2	2.2 ± 0.4	1.0
UV-3T3 T				
UV-I	**33 ± 11**	18 ± 2	1.5 ± 0.3	0.7
UV-II	**16 ± 10**	32 ± 3	3.3 ± 1.3	1.2
UV-III	**46 ± 12**	32 ± 2	2.6 ± 0.8	0.4

Comparable results were obtained in assays performed on cells grown for 12, 14, 23, 30, and/or 40 weeks after UV irradiation. Data are presented as the mean values ± SEM (n = 3–5). In selected studies, only two assays were performed; these data are therefore presented simply as mean values. Data in boldface type indicate the only significant differences between native and UV-irradiated cells.
*Differentiation is expressed as %.
†G_C, G_S, and G_N arrest are expressed as saturation density (cells per $cm^2 \times 10^3$).

can also induce the low frequency generation of completely transformed clones in specific cell types (24, 25). We therefore performed a series of experiments that were designed to assay the frequency of occurrence of UV irradiation-induced defects in the control of differentiation relative to the frequency with which UV-irradiated cells express a transformed morphology. To accomplish this goal, we again used the UV-I, UV-II, and UV-III 3T3 T proadipocyte cell lines. We first assayed the ability of native and UV-irradiated cells to grow in soft agar, but no colony formation was observed (data not shown). We therefore used another *in vitro* assay for the transformed phenotype, which in these studies was more sensitive—i.e., the focus forming assay (21, 24, 25). Our studies ask two questions. First, what is the frequency of generation of transformed foci in the original UV-irradiated cell lines and second, what is the frequency of generation of transformed foci in selected subpopulations of these cells? Such subpopulations were selected from morphologically normal areas that were evident in specimens subjected to the initial focus-forming transformation assays. With regard to this assay, it detects aberrant cells that grow to very high focal densities and express a spindle-shaped morphology—i.e., a type III transformed morphology (21, 24, 25). To isolate morphologically normal areas of such specimens, cells that grew on glass microchips were isolated and culturally expanded and then retested for the generation of type III foci. The results presented in Table 3 show that native 3T3 T proadipocytes show an extremely low transformation frequency in the focus-forming assay of 1×10^{-7}, whereas the original UV-irradiated 3T3 T cell lines showed a transformation frequency of $\approx 4 \times 10^{-5}$. This significantly increased

Table 3. UV irradiation induces minimal evidence of complete neoplastic transformation

Cell designation	Transformation frequency (type III focus-formation assay)	
	Original UV-irradiated cell populations	Selected UV-irradiated cell subpopulations
3T3 T	1.0×10^{-7}	—
3T3 T UV-I, -II, -III	3.8×10^{-5}	7.2×10^{-5}

Five morphologically nontransformed cell subpopulations were isolated from initial focus-forming assays. These five specimens were then reassayed with comparable results; therefore, the data have been averaged. Data presented are mean values; the standard error in the transformation frequency data for the original and selected cell subpopulations was 3.8 ± 0.1 and 7.2 ± 1.8, respectively. Statistical comparisons of these transformation frequencies were not significantly different by Student's t test ($P > 0.1$).

transformation frequency was also evident in five morphologically normal subsets of UV-irradiated cells that were isolated from the original foci formation assay. These observations strongly suggest that UV irradiation induces not only the high frequency development of defects in the control of differentiation (7×10^{-1}) but also an increased, yet low, frequency of spontaneous neoplastic transformation. In this regard, the development of neoplastically transformed foci occurred with 1/10,000th the frequency of UV-induced defects on the control of differentiation.

DISCUSSION

UV irradiation, an initiator of carcinogenesis (20, 21), is shown to inhibit the nonterminal and terminal differentiation of ≥70% of proadipocyte stem cells, whereas UV irradiation does not abrogate other cell cycle-dependent growth control processes. This effect is not only selective, it is also stable for >8 months in contrast to the effects of most other agents that affect proadipocyte differentiation, which are transient and/or reversible (22, 29). These observations support our concept of carcinogenesis (Fig. 1), which suggests that the development of defects in the control of differentiation may represent an important early event in carcinogenesis. This conclusion is further supported by the data showing that cells expressing differentiation defects also demonstrate an increased spontaneous transformation frequency relative to native 3T3 T cells.

Additional clinical and experimental observations, however, suggest that the initiation of carcinogenesis requires expression of other lesions in addition to the development of defects in the control of differentiation. The murine embryo cells that are used in many carcinogenesis studies, for example, are known to express chromosomal defects and additional biological defects (3, 10). Certain genetic diseases also predispose individuals to develop both preneoplastic lesions (30) and cancer (31, 32), and animal strains have also been developed that have a very high sensitivity to cancer-causing agents (33). These observations suggest that the first phase in the initiation of carcinogenesis involves the development of genetic instability (3, 34). In addition, we have suggested that aberrant commitment, the process that determines a stem cell's growth and differentiation potential, may also represent a very early event in carcinogenesis (9, 10, 12). The development of cellular immortality has also been proposed to represent a very early event in the transformation process (35); however, the demonstration that normal stem cells are immortal cast some doubt on this possibility (36).

The second phase of the initiation process according to our concept involves expression of overt defects in the control of differentiation. The role of such defects in carcinogenesis is supported by the studies reported in this paper and our other published reports (9–11). In addition, Sachs and co-workers (14, 37) reported that myeloid leukemia cells must show evidence of defective control of differentiation to be transformed, and Stanbridge and co-workers (13) also reported that before human cells can express the completely transformed phenotype *in vivo* they must express defects in the control of differentiation.

Finally, a third phase in the initiation of carcinogens may involve the acquisition of responsiveness to tumor-promoting agents by cells that express defects in their ability to differentiate. In this regard, both Mondal and Heidelberger (21) and Kennedy and co-workers (4, 5) reported that such cells must be exposed to additional carcinogenic agents before they could be promoted to form tumors *in vivo*. In support of these data, we previously reported that 3T3 T proadipocyte stem cells that possess intact differentiation control mechanisms are significantly less responsive to tumor-promoting agents than are differentiation-defective 3T3 cells (19).

The results of numerous additional studies are also consistent with the multiphase concept of the initiation of carcinogenesis. The most significant of these studies suggest that inactivation of purported cancer suppressor genes that may mediate the control of cellular differentiation must occur before transforming oncogenes can induce later phases in the process of carcinogenesis that generate tumorigenic cells (38–40).

We therefore suggest that the initiation of carcinogenesis requires the expression of multiple biological and molecular defects, one of which involves the development of defects in the control of cellular differentiation.

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