A Growth Factor-repressible Gene Associated with Protein Kinase C-mediated Inhibition of Adipocyte Differentiation

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Abstract. The conversion of determined adipoblasts to fully differentiated adipocytes requires appropriate environmental conditions. A strict dependence on cell confluence and a facilitation by glucocorticoid hormones have previously been described. We have found that agents that are capable of activating protein kinase C, such as basic fibroblast growth factor and phorbol esters, inhibit the differentiation of the adipogenic cell line TA1 without stimulating cell growth. Here we describe the sequence and characterization of a cDNA (clone 5) that detects an RNA, the expression of which is enhanced by glucocorticoids and increasing cell density. In contrast, activators of protein kinase C including basic fibroblast growth factor, phorbol esters, and synthetic diacylglycerols inhibit clone 5 gene expression. It appears that clone 5 expression is closely linked to environmental and hormonal factors that promote the differentiation of adipogenic cells.

The hormonal environment in which a stem cell finds itself is likely to be a major determinant controlling its decision to continue replicating or to express its terminally differentiated phenotype. The process of differentiation is generally reflected by the de novo synthesis of gene products that define the specific functions of that cell type. Thus erythroid cells will express globins, B lymphocytes will express immunoglobulins, and myocytes will express novel contractile proteins. Although the mechanisms by which the respective genes are activated remain uncertain, it seems that, as a rule, progenitor cells do not express functions characteristic of the differentiated phenotype while actively growing. It is clear that environmental or hormonal signals must participate in directing the cell to withdraw from the cell cycle and to progress towards the differentiated state. For example, addition of mitogenic stimuli such as serum to myoblasts stimulates these cells to continue cycling and at the same time represses all morphological and biochemical markers of myogenic differentiation (Buckingham, 1977; Devlin and Konigsberg, 1983; Nguyen et al., 1983; Olson et al., 1983). Removal of mitogens is followed by the expression of such markers. Despite the apparent clarity of such phenomena, both the nature of the molecular switch that controls the decision to differentiate and the mechanisms by which the cell senses and communicates its environmental status to that switch remain obscure (Levenson and Housman, 1981).

We have been particularly interested in understanding the mechanisms by which hormonal cues dictate the ability (or inability) of adipogenic stem cells to differentiate into mature adipocytes. TA1 adipoblasts were isolated from the mesenchymal stem cell line, 10T1/2, after treatment with 5-azacytidine (Chapman et al., 1984); Taylor and Jones (1979) first reported that 10T1/2 cells have the potential to differentiate into myocytes, chondrocytes, and adipocytes after exposure to this drug. When maintained at low density, TA1 cells resemble fibroblasts and do not express gene products characteristic of the mature adipocyte. However, when maintained at confluence spontaneous differentiation occurs, a process that can be markedly accelerated by treatment with glucocorticoid hormones or the nonsteroidal antiinflammatory drug, indomethacin (Chapman et al., 1985; Knight et al., 1987).

In contrast, if cells are maintained at low density, even in the presence of an inducing agent such as indomethacin, differentiation-specific genes are not activated (Knight et al., 1987). Moreover, continual refeeding drives TA1 cells to proliferate but even at high density prevents them from expressing differentiated functions (Chapman, 1986; see below). Thus it appears that only when the cells are at high density and not under continual mitogenic stimulation will they undergo the transition to the differentiated state.

In this manuscript we describe the isolation and sequence of a cDNA (clone 5) that detects a glucocorticoid-inducible RNA in TA1 cells, the abundance of which increases with increasing cell density and decreases dramatically after treatment with FCS or basic fibroblast growth factor (bFGF).

1. Abbreviations used in this paper: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; dC12, synthetic diacylglycerol. 1,2-dioctanoylglycerol; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; PKC, protein kinase C; SSPE, 0.18 M NaCl, 10 mM NaH2PO4, pH 7.7, 1 mM EDTA.
We find that bFGF, as well as other agents that activate protein kinase C (PKC) prevents TAI cells from differentiating and simultaneously inhibits the expression of clone 5 RNA. Suppression of clone 5 RNA expression by FGF or phorbol esters occurs in actively growing cells indicating that this effect is likely to be independent of the mitogenic activity of these factors. These studies suggest that activation of PKC prevents conversion of TAI cells to the differentiated phenotype without inducing cell growth. Finally, although the following hypothesis is not directly tested by this work, the pattern of regulation of clone 5 gene expression leads us to speculate that the clone 5 protein may be required, though not sufficient, for the triggering of adipocyte differentiation.

Materials and Methods

Cell Culture

TAI cells (Chapman et al., 1984) were grown in Eagle's basal medium (BME) (Gibco, Grand Island, NY) supplemented with 10% FCS (lot No. 50785, Irvine Scientific, Santa Ana, CA). All serum used was heat inactivated at 55°C for 30 min. Cultures were grown in either 35-mm wells (6-well plate) or 100-mm dishes at 37°C in a humidified incubator at 5% CO2 atmosphere.

Acidic fibroblast growth factor (aFGF) and bFGF were a gift of Dr. D. Gospodorowicz (University of California, San Francisco). Crude platelet-derived growth factor (PDGF) was a gift of Dr. L. T. Williams (University of California, San Francisco). Epidermal growth factor (EGF) and partially purified bFGF were from Collaborative Research, Inc. (Bedford, MA). The phorbol esters tetradecanoylphorbo1-13-acetate (TPA) and phorbol dibutyrate were from Sigma Chemical Co. (St. Louis, MO); they were dissolved in DMSO at 1,000× concentrations so that the final DMSO concentration was never >0.1%. The synthetic diacylglycerol, phorbol dibutyrate were from Sigma Chemical Co. (St. Louis, MO); they were dissolved in DMSO at 1,000× concentrations so that the final DMSO concentration was never >0.1%. The synthetic diacylglycerol, sn-1,2-diacylglycerol, sn-1,2-diacylglycerol (diC12) was from Avanti Polar Lipids (Birmingham, AL). diC12 was stored at 5,000× concentrations in DMSO. Dexamethasone and indomethacin (Sigma Chemical Co.) were made up in 95% ethanol and used at final concentrations of 1 µM and 125 nM, respectively.

RNA Isolation

Two methods were used for the isolation of RNA from TAI cells. Total cellular RNA was isolated using the method of Chirgwin et al. (1979). Briefly, cells were suspended in 4 M guanidinium-thiocyanate, 25 mM sodium citrate, 0.2% N-lauryl sarcosine, 0.2 mM 2-mercaptoethanol. The resulting cell lysate was layered on a 5.7 M cesium chloride solution and centrifuged at 80,000 g for 19 h. The RNA pellet was collected and its concentration was determined by reading absorbance at 260 nm. Cytoplasmic RNA was isolated using the method of Chirgwin et al. (1979). Briefly, RNA was dissolved in 20x SSPE and heated to 50°C for 10 min. The RNA concentration was never >0.1%. The synthetic diacylglycerol, sn-1,2-diacylglycerol, sn-1,2-diacylglycerol (diC12) was from Avanti Polar Lipids (Birmingham, AL). diC12 was stored at 5,000× concentrations in DMSO. Dexamethasone and indomethacin (Sigma Chemical Co.) were made up in 95% ethanol and used at final concentrations of 1 µM and 125 nM, respectively.

Analysis of RNA

5-10 µg of total RNA was treated with 2.2 M formaldehyde in 50% formaldehyde, 10 mM NaHPO4, pH7.0, at 55°C for 15 min. Samples were subjected to electrophoresis in a 1.4% agarose-formaldehyde gel containing 2.2 M formaldehyde, 20 mM morpholine propane sulfonic acid, pH 7.0, 5 mM sodium acetate, and 1 mM EDTA (Lehrach et al., 1977). The gels were stained with 10 µg/ml ethidium bromide and destained with several changes of 10 mM NaHPO4, pH 7.0. RNA was transferred to nitrocellulose using 20× SSPE (1× SSPE = 0.18 M NaCl, 10 mM NaHPO4, pH 7.7, 1 mM EDTA) as previously described (Danielsen et al., 1986). RNA was also analyzed using a "slot blot" method. 2-5 µg of total RNA was dissolved in 20× SSPE and heated to 50°C for 10 min. The RNA solution was then directly applied to the nitrocellulose using a Schleicher & Schuell manifold II apparatus (Schleicher & Schuell, Inc., Keene, NH). Blots were prehybridized in H buffer (50% formamide, 5× SSPE, 0.1% SDS, 2× Denhardt's solution) and then washed at 50°C for 20 min. Filters were then subject to prehybridization and hybridization as above, but the RNA probe was used at 10 µg/ml. Filters were then washed at 50°C. Filters were then subject to prehybridization and hybridization as above, but the RNA probe was used at 10 µg/ml. Filters were then washed at 50°C. Filters were then subject to prehybridization and hybridization as above, but the RNA probe was used at 10 µg/ml. Filters were then washed at 50°C. Filters were then subject to prehybridization and hybridization as above, but the RNA probe was used at 10 µg/ml. Filters were then washed at 50°C. Filters were then subject to prehybridization and hybridization as above, but the RNA probe was used at 10 µg/ml. Filters were then washed at 50°C.
Clone 5 RNA Levels Are Regulated by Cell Density and Serum Factors

The abundance of clone 5 RNA in TA1 adipoblasts increases as cells become more dense. For example, cells harvested 5 d before confluence (day −5) contain approximately four- to sixfold less clone 5 RNA than do cells at day 0 (Fig. 2 A). Similarly, when TA1 cells are plated at various dilutions and harvested 48 h later, the lower density cells contain less clone 5 RNA (Fig. 2 B). This could reflect the secretion of an autocrine factor that induces clone 5 RNA and/or the depletion of a component in the growth medium that suppresses clone 5 RNA expression. A strong suggestion that the latter is true is afforded by the observation that when cells are fed fresh medium, the levels of clone 5 RNA decrease fivefold within the subsequent 24-h period. Maximal suppression occurs 16-18 h after feeding and a return to the prefed levels of clone 5 RNA is seen within 48-72 h (not shown).

To test the role of various components of the growth medium on clone 5 RNA expression we established a standardized experimental protocol: TA1 cells were plated at day −5 or −4 and 3 d later the cells were either refed (removal of old medium followed by replacement with fresh medium containing BME + 10% FCS) or treated by addition of test substances directly to the 3-d-old medium. Control dishes were either left undisturbed or refed with 3-d-old medium. Total RNA was harvested 24 h later and analyzed for clone 5 RNA levels. As seen in Fig. 3 A, the effect of refeeding could be duplicated completely by addition of fresh serum.

To determine what factor(s) in serum might inhibit clone 5 expression, either modified serum or purified serum components were added to TA1 cells in the standardized assay described above. Initial experiments (not shown) indicated that whereas dialyzed serum suppresses clone 5 RNA expression, charcoal-extracted serum and insulin (at 1 μM) do not. Fig. 3 A also summarizes the various combinations of components and purified factors that we tested. Both bFGF and aFGF as well as PDGF maximally (approximately fivefold) suppress clone 5 RNA levels whereas EGF either in the presence or absence of insulin does not. As noted above, the suppression of clone 5 RNA by serum is transient; therefore, we tested whether the recovery of clone 5 RNA could be attributed to inactivation of FGF- or PDGF-like material in serum. Fresh medium (BME + 10% FCS) was incubated in tissue culture dishes (at 37°C, 5% CO2) without cells for 24 or 48 h. As seen in Fig. 3 B preincubated medium loses its ability to suppress clone 5 RNA expression, thereby substantiating the notion that it is a labile factor in serum that is responsible for the refeeding effect we have described.

In light of these results, one might question conclusions derived from the density experiment described earlier (Fig. 2 B). The higher levels of clone 5 RNA in the denser cultures could be attributed to the more rapid consumption of growth factors by these cells, followed by an earlier recovery from the inhibitory effect on clone 5 expression. To dissociate the effect of serum factors on clone 5 expression from the effects of cell density, we performed the following experiment: TA1 adipoblasts at day −1 from two dishes were seeded into twenty dishes, half of which received fresh medium and the other half “inactivated” medium which had been incubated for 48 h as described above. As shown in Fig. 4, the level of clone 5 RNA in these cells is reduced fivefold in both cases 24 h after seeding. For the cells seeded into fresh medium, this reduction in clone 5 RNA could be attributed to serum factors. For the cells seeded into aged medium, the effect must be attributed to the effects of change in cell density due to trypsinization, since the medium alone is incapable of affecting clone 5 levels. Moreover, we see that the level of clone 5 RNA increases as the cells become more dense (at 48 and 72 h). Once again, this cannot be attributed to a recovery from inhibition by serum. By 72 h, the cells have
The active growth of TA1 cells. Thus it appears that the suppression of clone 5 expression is not simply due to mitogenic stimulation of the cells.

Figure 3. Inhibition of clone 5 RNA accumulation by medium components and purified factors. TA1 cells were treated on day −2 or −1 with the indicated agents. 24 h later, cytoplasmic RNA was isolated from the cells and quantitatively analyzed for clone 5 RNA levels using the “slot blot” technique described in Materials and Methods. In all cases, cells were seeded on day −5 and not refed unless indicated below. (A) The added components were 10% FCS, aFGF at 10 ng/ml, bFGF at 1.0 or 0.1 ng/ml, PDGF at 10 ng/ml, 100 ng/ml EGF in the presence or absence of 1 μM insulin, or untreated cells (NT). (B) On day −2, medium was removed from TA1 cells, which were refed with medium pretreated for 24 or 48 h (see text), or with the same medium which was just removed from the dish. RNA was analyzed as above. (C) Inhibition by direct activators of PKC. On day −2 or −1, the indicated compound was added to the cells and RNA isolated 24 h later. The tested agents were 1 μM phorbol dibutyrate (PDB) or TPA at the concentrations shown. In the case of dICs, cells were treated initially with 50 μM (50/20) or 2 μM (2/2) dICs, and then treated six more times, every 2 h with 20 μM (50/20) or 2 μM (2/2) dICs. Cells were harvested 18 h after the initial treatment and analyzed for clone 5 RNA as described above.

Figure 4. Effect of cell density on clone 5 expression uncoupled from the refeeding effect. TA1 cells (not fed since seeding at day −5) at day −1 were split into 10 dishes, fed with either fresh medium or medium incubated at 37°C in 5% CO2 without cells for 48 h (see text). RNA was isolated from the initial day −1 cells (○ at day 0 = 100) and 24, 48, and 72 h later from the cells seeded in fresh (●) or aged (○) medium. Clone 5 levels were analyzed as in Fig. 3.

A Role for PKC in Control of Clone 5 Gene Expression

Both bFGF and PDGF have been shown to induce turnover of phosphatidylinositol thereby generating diacylglycerol and inositol triphosphate (Habenicht et al., 1981; Tauda et al., 1985). Since many of the actions of these hormones seem to be mediated by activation of PKC with diacylglycerol (Berridge, 1984; Nishizuka, 1984) we tested whether this might also be true of clone 5 RNA expression. Addition of exogenous activators of PKC, such as the phorbol esters TPA or phorbol dibutyrate to TA1 cells led to a dramatic reduction of clone 5 RNA levels (Fig. 3 C). The concentration of TPA required to suppress clone 5 RNA expression is in the range reported to be sufficient to activate PKC (Nishizuka, 1984). Finally, the use of dICs, when applied at dosages known to activate PKC in other systems (Ebeling et al., 1985, Ganong et al., 1986) also reduces clone 5 expression to the same extent as serum (Fig. 3 C). Thus we surmise that the ability of FGF and PDGF to inhibit clone 5 gene expression is most likely due to the activation of PKC, though other signal transduction pathways may also play a role.

Growth Factor Inhibition of TA1 Cell Differentiation

TA1 adipoblasts will not differentiate if maintained at low density (Knight et al., 1987) or if chronically stimulated with fresh complete medium (Chapman, 1986). Under the latter situation the cells continue to proliferate and do not express any markers of the differentiated phenotype. To test whether any of the growth factors that suppress clone 5 gene expression interfere with differentiation, TA1 cells were induced to differentiate by addition of indomethacin at day 0. In the absence of any further additions >95% of the cells differentiated within 3 d as determined by detection of accumulated lipids with oil red O (not shown) and induced active expression of clone 28 RNA (Fig. 5 A). However, addition of 10% FCS, bFGF, or TPA every 24 h suppresses the induction of clone 28 RNA at day 3 (Fig. 5 A) and morphological differentiation of the cells (not shown). In contrast, neither EGF nor low concentrations of serum have much effect on the accumulation of clone 28 RNA. By slightly altering the differentiation assay, we can accentuate the inhibitory effects of bFGF and TPA on adipocyte differentiation. In the experiment shown in Fig. 5 B, the cells were refed 1 d before confluence. Indomethacin was added on day 0, along with bFGF or TPA. The daily addition of bFGF (1.0 ng/ml) or
growth factors or TPA cannot be attributed to mitogenic stimulation through the cell cycle.

Figure 5. Inhibition of adipogenic differentiation by serum and growth factors. (A) Cultures of TA1 cells (not fed since seeding at day -5) were reared and treated with indomethacin on reaching confluence (day 0). In addition, the cells were treated with either 10% serum, 1% serum, bFGF at 10 ng/ml, 1 μM TPA, or no treatment (NT). These additions (but not indomethacin) were repeated on days 1 and 2. RNA was isolated on day 3, and the extent of differentiation determined by analyzing clone 28 levels (as in Fig. 1). (B) TA1 cells (not fed since seeding at day -5) were reared on day -1 and treated with indomethacin upon reaching confluence (day 0). Highly purified bFGF at 1 ng/ml or 0.1 ng/ml, or TPA at 100 or 1 nM were added along with the indomethacin, and repeated on days 1 and 2. RNA was isolated on day 3 and the extent of differentiation determined by analyzing the levels of clones 1 and 28.

TPA (100 nM) significantly suppressed the expression of the RNAs corresponding to clone 28 and clone 1 (another adipocyte-specific cDNA used for monitoring differentiation [Chapman et al., 1984]). When applied at concentrations that do not inhibit clone 5 expression (Fig. 3), bFGF (0.1 ng/ml) and TPA (1 nM) did not affect adipogenesis. Thus those agents that (at appropriate concentrations) inhibit clone 5 RNA expression are also those that suppress differentiation of TA1 cells.

The inhibition of differentiation by the various agents described above could be explained if the adipoblasts are stimulated to reenter the cell cycle and proliferate. In the case of daily additions of serum, this appears to be what happens. At the time of harvest, serum-treated cultures consistently have two- to threefold the number of cells per dish (2.6 × 10⁶ cells/plate) as the untreated cells (1.1 × 10⁶ cells/plate). In contrast, TA1 cultures treated with bFGF or TPA have the same number of cells as the untreated cultures (1.1 × 10⁶ and 0.9 × 10⁶ cells/plate, respectively). Thus, as is true for suppression of clone 5 RNA, inhibition of differentiation by growth factors or TPA cannot be attributed to mitogenic stimulation through the cell cycle.

**Sequence of the Clone 5 cDNA**

To characterize the clone 5 gene product, we rescreened the TA1 adipocyte cDNA library prepared by Chapman et al. (1984) and isolated a clone containing an insert of ~1,000 base pairs (the mRNA is ~1.1 kb in length). The inserts were subcloned into a plasmid vector and the nucleotide sequence was determined (Fig. 6) by the method of Sanger et al. (1977). An open reading frame of 244 amino acids is revealed no significant homologies. However, the translated amino acid sequence, especially when compared to the National Biomedical Research Foundation protein database, revealed significant homology between the clone 5 open reading frame and ribitol dehydrogenase of *Enterobacter aerogenes* (Morris et al., 1974) (Fig. 7). The most striking homology resides between amino acids 10 and 35, a region that exhibits a resemblance to the NAD-binding domain of other dehydrogenases and to ATP-binding proteins. Particularly noteworthy are the residues 14–21 (GAGKGIGR) which are followed by an Arg residue.

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2. These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number X07411.

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It is only recently, however, that the ATP-binding domains of various protein kinases and ATPases (Kamps et al., 1984).

The effect of growth factors on differentiation is not limited to adipogenic lines. The differentiation of many myogenic cells, such as MM14 myoblasts, is also inhibited by bFGF or PDGF (Chapman et al., 1987). Hayashi et al. (1981) demonstrated that the addition of highly purified bFGF or PDGF to 3T3-L1 adipoblasts inhibited adipogenic conversion. On the other hand, EGF, even at high concentration, had no effect on 3T3-L1 differentiation. Moreover, the authors noted that bFGF and PDGF, both mitogens of 3T3 cells, also had little effect if any on cell multiplication under the assay conditions used. Similarly, Diamond et al. (1977) and Shimizu et al. (1983) have shown that TPA treatment of 3T3 adipocytes also inhibits adipogenesis. These authors also found that the addition of TPA to 3T3 preadipocytes did not force those cells to divide. Thus the mechanisms by which these factors inhibit differentiation appear to be independent of their effects on cell proliferation.

The effect of growth factors on differentiation is not limited to adipogenic lines. The differentiation of many myogenic cell lines has been shown to be sensitive to FGF. As an example, Clegg et al. (1987), while studying the FGF-mediated inhibition of MM14 myoblast differentiation, found that in the absence of serum, FGF would block myogenesis without inducing cell replication, causing the cells to stop cycling in

12 amino acids later by lysine; this motif is characteristic of the ATP-binding domains of various protein kinases and ATPases (Kamps et al., 1984).

**Discussion**

That hormones control aspects of differentiation has long been assumed to be true. It is only recently, however, that well-defined systems have become available to study the molecular and biochemical details associated with differentiation. In the case of steroid hormones, whose receptors act as regulators of gene transcription (Yamamoto, 1985), effects on differentiation are presumed to depend on activation (or repression) of regulatory genes (Ringold, 1985). The hormonal effect is associated with the preconfluent cultures of TAI cells show little tendency to differentiate, even in the presence of strong inducers of differentiation. Thus factors associated with high cell density are critical in the decision to differentiate. Similarly, chronic mitogenic stimulation acts as a negative regulator of differentiation. As shown here and elsewhere (Chapman, 1986), confluent cultures of TAI cells stimulated daily by serum continue to proliferate and do not undergo adipogenesis, even in the presence of indomethacin. The inability of cells to differentiate while mitogenically stimulated is not peculiar to adipocytes and may well be a general phenomenon.

In this paper we have described several characteristics of clone 5 RNA expression that suggest the presence of this gene might play a regulatory function in the conversion of adipoblasts to adipocytes. In particular, environmental factors that stimulate adipogenic differentiation such as glucocorticoids and high cell density increase the expression of clone 5 RNA. Moreover, factors that inhibit clone 5 expression such as bFGF or phorbol esters also inhibit adipogenic differentiation.

Some aspects of the control of clone 5 gene expression by growth factors deserve particular attention. First, the direct inhibition of gene expression by bFGF and PDGF (i.e., not separable from indirect effects on differentiation-dependent gene expression) has not, to the best of our knowledge, been actively studied. Our results suggest that, as is the case for PDGF-mediated induction of c-myc and c-fos (Coughlin et al., 1985; Kaibuchi et al., 1986), the repression of clone 5 expression by bFGF or PDGF is mediated by activation of PKC. Second, the effects of bFGF or PDGF on clone 5 expression appear to be separable from their mitogenic activity. Suppression of clone 5 gene expression by these growth factors (or TPA) occurs in TAI cells already stimulated to grow by serum mitogens. Similarly, the inhibition of differentiation by these same agents occurs under conditions in which they do not stimulate cell growth. It therefore seems clear that the inhibition of TAI cell differentiation by bFGF or TPA cannot simply be ascribed to the mitogenic activity of these agents.

The finding that known mitogens inhibit adipogenic differentiation without inducing cell proliferation is not unique to the TAI cell type. Similar results have been observed with another adipogenic cell line, 3T3-L1. Hayashi et al. (1981) demonstrated that the addition of highly purified bFGF or PDGF to 3T3-L1 adipoblasts inhibited adipogenic conversion. On the other hand, EGF, even at high concentration, had no effect on 3T3-L1 differentiation. Moreover, the authors noted that bFGF and PDGF, both mitogens of 3T3 cells, also had little effect if any on cell multiplication under the assay conditions used. Similarly, Diamond et al. (1977) and Shimizu et al. (1983) have shown that TPA treatment of 3T3 adipocytes also inhibits adipogenesis. These authors also found that the addition of TPA to 3T3 preadipocytes did not force those cells to divide. Thus the mechanisms by which these factors inhibit differentiation appear to be independent of their effects on cell proliferation.

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