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# TPA Induced Expression and Function of Human Connexin 26 by Post-Translational Mechanisms in Stably Transfected Neuroblastoma Cells

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*ABSTRACT.* **Connexin 26 (Cx26) has been proposed to be a tumor suppressor gene and its expression may modulate development, cell growth and differentiation in various tissues, including the brain. 12-O-tetradecanoylphorbol-13-acetate (TPA) may serve as either tumor promoter (in mammary gland amd skin) or as a differentiating agent (in neuroblastoma and leukemic cells) and may also modulate expression, function and phosphorylation of gap junctions. In this study, to determine the effects of TPA on Cx26 expression and its function in neuroblastoma, we transfected N2A mouse neuroblastoma cells (which are gap junction deficient) with the coding region of human Cx26 gene (which lacks TPA response elements) and examined the changes of expression and function of Cx26 following 10 nM TPA treatment. Individual clones of transfectants stably expressed distinct levels of exogenous Cx26 as judged by Northern and Western blots, immunocytochemistry and electrophysiological recordings. Cx26 channels displayed unitary conductances of about 140–155 pS. Increase of Cx26 expression following TPA treatment was markedly observed using immunocytochemistry and Western blots of membrane fractions although it was not detected in Northern or Western blots of whole cells. This increase in Cx26 expression in the plasma membrane was accompanied by an increase of function as evidenced in measurements of junctional conductance. These results suggest that induction of exogenous Cx26 in neuroblastoma cells by TPA treatment is controlled by post-translational mechanisms.**

**Key words:** gap junction/connexin/TPA/coupling/phorbol esters

Gap junction channels, composed of proteins termed connexins, mediate reciprocal exchange of ions and small molecules with Mr less than 1,000 Da, including second messengers, such as cyclic AMP, $IP_3$ and $Ca^{2+}$ between adjacent cells (5, 15, 22, 23). Gap junctional intercellular communication is thought to play a crucial role in development, cell growth and cell differentiation (2, 3, 19, 30). Connexin 26 (Cx26) is expressed in various tissues and organs including hepatocytes, placenta, mammary gland, skin, pancreas, endometrium, yolk sac and brain (5, 7). Cx26 has been proposed to be a tumor suppressor gene, on the basis of subtractive hybridization using normal and malignant human mammary epithelial cells (17). Furthermore, it is also reported that mice lacking Cx26 die in utero, presumably as a result of failure of placental formation or function (9) and in several human kindreds with nonsyndromic hereditary deafness, Cx26 appears to be the culprit gene (12, 32).

12-O-tetradecanoylphorbol-13-acetate (TPA) may serve as either tumor promoter or differentiation agent, depending on cell type (1, 20, 31). Furthermore, TPA treatment modulates expression, function and phosphorylation of gap junctions in numerous cell types, which in cells expressing Cx43 has been associated with phosphorylation of the gap junction protein (4, 16, 21). Cx26 is unusual in that it has the shortest carboxyl tail of the connexin family members and is not phosphorylated (22, 33). Recently, it was reported that Cx26 expression in human mammary epithelial cells induced by TPA treatment is controlled at the level of transcriptional modification (18). Kiang *et al.* have cloned and

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sequenced that 5′ portion of human Cx26 gene and the promoter region contains six GC boxes, two GT boxes, a TTAAAA box and a TPA-induced DNase I hypersensitivity (DH) region (13, 28), providing a site by which TPA may exert transcriptional control over Cx26. In order to examine the mechanism of TPA enhanced expression of Cx26, we transfected N2A mouse neuroblastoma cells (which are gap junction deficient) with the coding region of human Cx26 gene (which lacks TPA/TPA like-response elements) and examined changes of expression and function of the Cx26 following TPA treatment. These findings indicate that the expression and function of human Cx26 exogenously expressed in neuroblastoma cells were markedly induced by TPA treatment through post-translational mechanisms.

## *Materials and Methods*

### *DNA construction and transfection*

A fragment containing nucleotide 1 to 680 of the human connexin 26 (Cx26) cDNA (13) was subcloned into the expression vector pRC/CMV (Invitrogen, Carlsbad, CA, USA) at the HIND III-XBAI restriction site. N2A mouse neuroblastoma cells (obtained from ATCC, and subcloned by dilution to generate a parental cell line that expressed only minimal endogenous Cx45) were transfected with 2 $\mu$g of DNA using the LIPOfectamine regent (GIBCO BRL, Gaithersberg, MD, USA). After 48 h, the cells were transferred to selection medium containing 800 $\mu$g/ml G418 (Sigma, St. Louis, MO, USA). When surviving clones were large enough to be detected visually, they were transferred to separate dishes for expansion. Initial analysis of the RNA and the immunostaining from a number of clones led us to choose four. All cell cultures were maintained with DMEM medium containing 4% FBS and antibiotic at 37°C in 5% $CO_2$/95% air incubator. Furthermore, some of the human Cx26 transfectants were treated with 10 nM TPA (Sigma) for 6 h, 24 h or 48 h.

### *RNA isolation and Northern blot analysis*

Total RNA was extracted from the cells using TRIzol (BRL, Grand Island, UY, USA). For Northern blot, 10 $\mu$g of total RNAs was loaded on 1% agarose gels containing 0.5 mg/L ethidium bromide. Gels were capillary-blotted in 20 × standard saline citrate (SSC) onto a nylon membrane (Hybond-N; Amersham Corp., Buckinghamshire, UK) and fixed by ultraviolet light. For detection of Cx26 mRNA, the membranes were prehybridized for 30 min at 68°C in QuickHyb solution (Stratagene, La Jolla, CA, USA), and then hybridized for 1 h at 68°C in the same solution with a $^{32}$P-labeled cDNA probe for rat Cx26 (33). The membranes were washed twice in 2 × SSC buffer containing 0.1% SDS at RT and once in 0.1 × SSC buffer containing 1% SDS at 60°C before exposure to film.

### *Western blot analysis*

For Western blot of whole cells, the dishes were washed with PBS twice and 300 $\mu$l of the buffer (1 mM $NaHCO_3$ and 2 mM PMSF) was added to 60 mm dishes. The cells were scraped and collected in Eppendorf tubes and then sonicated for 10 sec. Protein concentration in the sonicates were measured using a protein assay kit (Pierce Chemical Co., Rockford, IL, USA). Twenty $\mu$g of protein of each sample per lane was applied and separated by electrophoresis in 12% SDS-polyacrylamide gel (Bio-Rad, Richmond, CA, USA). After electrophoretic transfer to a nitrocellulose membrane (Bio-Rad), the membrane was saturated overnight at 4°C with a blocking buffer (25 mM Tris, pH 8.0, 125 mM NaCl, 0.1% Tween 20 and 4% skim milk) and incubated with polyclonal anti-Cx26 antibody (Zymed Laboratories Inc., San Francisco, CA, USA) at RT for 1 h. The membrane was incubated with a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) at RT for 1 h and the detection was carried out using an enhanced chemiluminescence (ECL) Western blotting system (Amersham Corp., Buckinghamshire, UK).

In addition, we also performed Western blot of the membranes extracted with 20 mM NaOH (11) to examine changes of Cx26 expression on the plasma membranes. The dishes were washed with PBS twice and 300 $\mu$l of the buffer (20 mM NaOH and 1 mM $Na_2CO_3$ and 2 mM PMSF) was added to 60 mm dishes. The cells were scraped and collected in Eppendorf tubes and then sonicated for 10 sec. The insoluble pellet was collected after centrifugation at 15,000 rpm for 15 min, washed once with the same solution, and then washed once with 1 mM $Na_2CO_3$. The insoluble pellets were then dissolved in Laemmli sample buffer with 100 mM DTT.

### *Immunofluorescence Microscopy*

The cells plated on glass cover slips were fixed with cold absoluted acetone for 10 min. After rinsing with phosphate-buffered saline (PBS), some coverslips were incubated with an anti-Cx26 polyclonal antibody at RT for 1 h and then were incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit IgG (DAKO, Copenhagen, Denmark) at RT for 1 h. All samples were examined with an epifluorescence photomicroscope.

### *Electrical coupling assay*

The dual whole cell voltage clamp technique (26) with patch pipettes (29) was used to measure macroscopic junctional conductance, voltage sensitivity of junctional conductance, and unitary conductances of junctional channels between cell pairs. Junctional currents ($I_j$) were measured as the currents recorded in one cell in response to 300 msec 2 mV pulses ($V_j$) applied to the other cell; junctional conductance ($g_j$) was cal-

culated as $I_j/V_j$. For single channel recordings, cell pairs with low $g_j$ were chosen. Single channel currents ($\gamma_j$) were detected as abrupt, step-like transitions occurring stimultaneously in both cell current traces when transjunctional voltages were applied to one cell, amplitudes of mainstate and substate unitary currents were determined as slope conductances from ramp protocols (±100 mV) and from dwell time histograms in response to sustained $V_j$ ("gap-free recording" in Fetchex) (25).

### *Statistical analysis*

Results are given as means±SEM. Differences between groups were tested by the two-tailed Student's t test for unpaired data.

## *Results*

### *Expression levels of mRNA and protein of Cx26 in human Cx26 transfectants*

In order to study the expression and function of Cx26, we stably transfected N2A cells with vector containing the coding region of human Cx26 cDNA as described in *Materials and Methods*. Numerous clones surviving selection were picked and grown for additional studies. Of these clones, four showed strong expression of mRNA with a size expected for the exogenous transcript (0.7 kb) as determined by Northern blot analysis. A representative Northern blot for Cx26 is shown in Figure 1A, indicating that clones 1, 2, 3, 4 exhibit exogenous transcript, whereas it was not expressed in parental N2A cells; mouse liver expressed endogenous Cx26 mRNA (2.4 kb). Furthermore, we performed Western blot for Cx26 using whole cells of clones 1, 2, 3, 4 which expressed Cx26 mRNA (Fig. 1B). In all clones the expression of Cx26 protein was observed with the same size as endogenous Cx26 protein in mouse liver, whereas it was not expressed in parental N2A cells. Immunostaining for Cx26 in the transfectants revealed Cx26 immunoreactive products in the cytoplasm of almost all cells and on cell borders of some cells (Fig. 2A), while in N2A parental cells the immunoreactive products were never observed.

### *Changes of mRNA and protein of Cx26 in human Cx26 transfectants treated with TPA*

In order to examine whether TPA treatment affected Cx26 expression in transfectants by TPA, we treated these cells with 10 nM TPA for 6, 24 or 48 h and performed immunocytochemistry and Northern and Western blot analyses using whole cells and Western blot of cell membranes extracted with NaOH. By immunocytochemistry for Cx26, a marked increase of Cx26 immunoreactive products was observed in the transfectants following 48 h exposure to 10 nM TPA compared to untreated transfectants, and the immunoreactive products were seen on cell borders of most of the treated transfectants (Fig. 2), while at 6 and 24 h, the changes were unclear. In Northern blot (Fig. 3A) and Western blot using whole cells (Fig. 3B) of the transfectants following 48 h exposure to 10 nM TPA, no change in the amount of Cx26 mRNA or protein was observed. However, when we performed Western blot analyses using membranes extracted with NaOH from the same samples, the expression of Cx26 protein was clearly shown to be increased in transfectants following 48 h exposure to 10 nM TPA compared to membranes extracted from untreated transfectants (Fig.

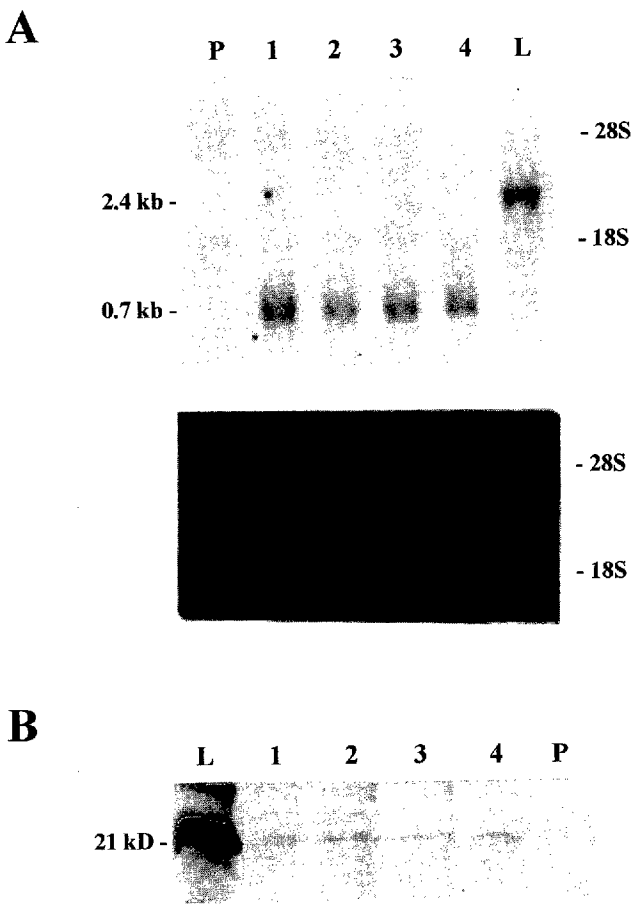


**Fig. 1.** Northern blot analysis for Cx26 mRNA (A) and Western blot analysis for Cx26 protein (B) in human Cx26 transfected N2A cells. P: parental N2A cells, 1–4: separately isolated transfectants, L: mouse liver. A (top panel): Expression of Cx26 mRNA corresponding to the exogenous 0.7 kb transcript was observed, whereas mouse liver expressed endogenous Cx26 mRNA (2.4 kb). Bottom panel in A illustrates 18S and 28S rRNA in ethidium bromide-stained gels, indicating comparable loading of these samples. B: Expression of Cx26 protein was observed in all transfectants and mouse liver, while it was not detected in parental N2A cells.

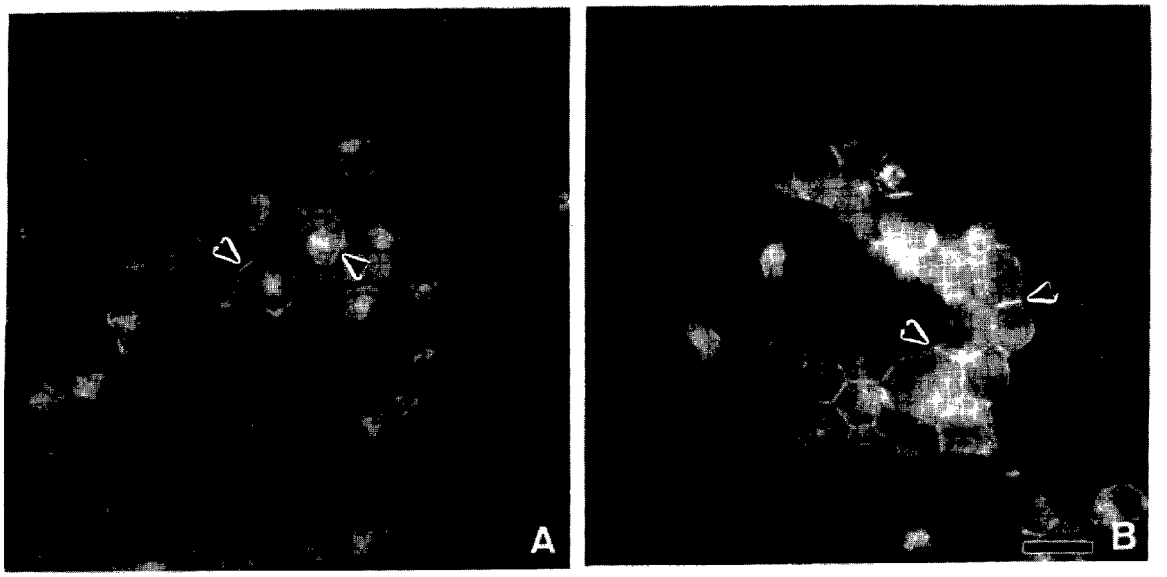


**Fig. 2.** Immunocytochemistry for Cx26 in human Cx26 transfected N2A cells (clone 2) untreated (A) or treated with 10 nM TPA (B) for 48 h. In some of untreated transfectants, Cx26-immunoreactive products were observed in the cytoplasm and at cell borders (arrowheads in A). The immunoreactive products at cell borders were increased by TPA treatment (arrowheads in B). In both treated and untreated cell, diffuse cytoplasmic Cx26 staining was also observed. Bar: 10 $\mu$m.

3C).

### *Changes of functional coupling in human Cx26 transfectants treated with TPA*

In order to examine whether functional coupling was induced in the Cx26 transfectants by TPA treatment, we measured junctional conductance between cell pairs using the dual whole cell voltage clamp technique. In nutreated transfectants, junctional conductance was low enough to evaluate single channel currents; one such recording is illustrated in Figure 4A. An all points histogram performed on this recording indicated that unitary junctional conductance was 153 pS. Unitary conductances recorded in other cell pairs ranged from 140 to 155 pS. In contrast, parental N2A cell pairs very rarely coupled, with only a few cell pairs exhibiting low $g_j$ with unitary conductances of about 30 pS (data not shown). Figure 4B shows that junctional conductance significantly increased in the transfectants following

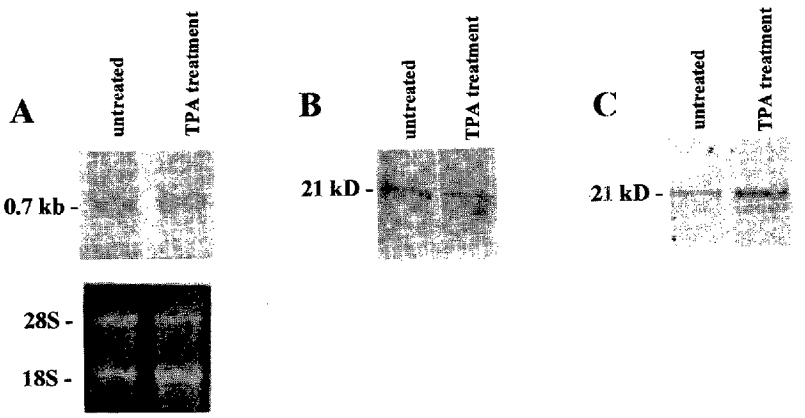


**Fig. 3.** Northern blot analysis for Cx26 mRNA (A) and Western blot analysis for Cx26 protein (B, C) in human Cx26 transfected N2A cells (clone 2) untreated or treated with 10 nM TPA for 48 h. (B): whole cells, (C) membranes extracted with NaOH. In Northern blot (A) and Western blot using whole cells (B) of the transfectants treated with TPA, no change in amount of Cx26 mRNA or proten was detected. Expression of Cx26 protein was markedly increased in the membranes extracted from the transfectants treated with TPA compared to that of untreated transfectants (C). Bottom panel in A illustrates gel loading.

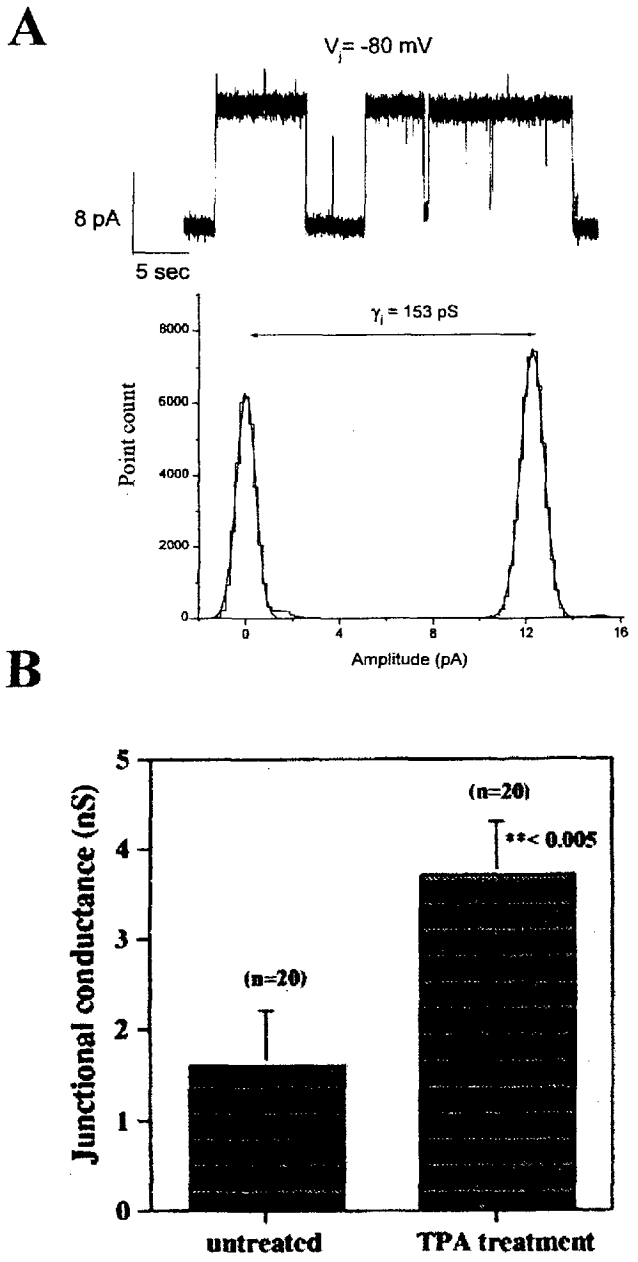


**Fig. 4.** Electrophysiological analysis of human Cx26 transfected N2A cells (clone 2). (A) In untreated transfectants, unitary gap junction channel activity recordings (upper panel) and amplitude histogram for the recording (lower panel) are shown. (B) Junctional conductances are shown in the transfectants untreated or treated with 10 nM TPA for 48 h. Junctional conductance in the transfectants was significantly increased by TPA treatment.

48 h exposure to 10 nM TPA compared to that of untreated transfectants (untreated transfectants: $1.6 \pm 0.6$ nS, TPA treated transfectants: $3.6 \pm 0.6$ nS; $P < 0.005$).

## *Discussion*

Although most connexins are phosphoproteins (10, 24, 27), Cx26 has the shortest carboxyl tail of the connexin family members and is not phosphorylated (33). Experiments using a cell-free system have shown that Cx26 can insert into membranes post-translationally (34), and the formation of Cx26 homotypic channels on the membranes has been suggested to follow an alternative non-classical trafficking pathway that bypasses the Golgi system (8). We previously reported that perfusion of female rat liver resulted in a rapid specific appearance of Cx26 in the plasma membrane without an increase in total protein or mRNA levels, suggesting the possibility that Cx26 expression at the plasma membrane might be controlled by post-translational mechanisms (14). TPA treatment modulates expression and function of gap junctions in numerous cell lines (4, 16, 21), including mammary tumor cell lines where Cx26 is selectively induced (17). Induction of Cx26 expression in human mammary epithelial cells by TPA treatment is controlled by transcriptional modification (18), which has been attributed to activation of a TPA-induced DNase I hypersensitivity (DH) region containing two TRE-like TGAT/ATCA elements (13, 28). In this study, to examine whether TPA might also regulate Cx26 expression and function through post-transcriptional mechanisms, we transfected N2A mouse neuroblastoma cells (which are gap junction deficient) with the coding region of human Cx26 gene (which lacks TPA response elements) and examined the changes of expression and function of Cx26 following 10 nM TPA treatment. Individual clones of transfectants stably expressed distinct levels of exogenous Cx26 as judged by Northern and Western blots, immunocytochemistry and electrophysiological recordings. Consistent with reports on the rodent Cx26 isoform (6), the channels in transfectants displayed unitary conductances of about 140–155 pS (Fig. 4A), and were only weakly voltage sensitive (data not illustrated, but note long open times for channels in Fig. 4A at $V_j = -80$ mV). Cx26 expression following TPA treatment was markedly enhanced as evaluated by immunocytochemistry and Western blots of membrane fractions but changes were not observed in Northern and Western blots of whole cells, indicating that TPA caused a change in distribution of Cx26 but not its abundance. This increase in Cx26 expression was accompanied by an increase of function as evidenced in the electrical coupling assay. We concluded from these results that induction of Cx26 in neuroblastoma cells by TPA treatment is controlled by post-translational modification. Although TPA is a potent tumor promoter (1, 31), it may also function as a differentiating agent (20). Although the mechanisms responsible for Cx26 translocation to the plasma mem-

branes that we have observed in transfected neuroblastoma cells by TPA treatment requires further study, it is possible that altered Cx26 expression may provide a marker of neuroblastoma differentiation. Alternatively or in addition, further analyses of the mechanisms underlying the TPA effects may provide insight into processes responsible for differentiation of these cells.

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