Signal Transduction Pathways Underlying the Expression of Tissue Factor and Thrombomodulin in Promyelocytic Cells Induced to Differentiate by Retinoid Acid and Dibutyryl cAMP

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Key words
Cell differentiation, promyelocytic cells, signal transduction, tissue factor, thrombomodulin

Summary
Acute promyelocytic leukaemia (APL) may be associated with disseminated intravascular coagulation, as a result of increased tissue factor (TF) expression and reduced thrombomodulin (TM) expression by APL blast cells. During retinoid acid (RA) and dibutyryl cAMP (dbcAMP)-induced differentiation of the APL cells, there is a marked up-modulation of both the protein kinase A (PKA) and C (PKC) activities. In order to further assess whether these kinases are intimately associated with both the differentiation process and the regulation of TF and TM expression, we have correlated the modulation of their respective pathways with the extent of differentiation and modulation of these cellular receptors. NB4 cells were incubated with all-trans-RA (ATRA) or dbcAMP for up to 48 h. The contribution of phospholipase C (PLC), inositol phosphate (IP), PKC and PKA in the expression of CD11b, TF and TM was studied by the use of specific inhibitors. Myo-inositol uptake and PKC activity increased in cells induced to differentiate by ATRA but the retinoid did not affect cAMP levels or PKA activity. Under treatment with dbcAMP, PKA activity was increased while inositol uptake and PKC activity remained unchanged. Our results show that the effects of ATRA and dbcAMP on promyelocytic cells are closely related, respectively, to the PLC/IP/PKC and the cAMP/PKA pathways. In cells induced to differentiate by ATRA, CD11b expression seems more closely related to inositol uptake than to PKC activity while the expression of TF and TM show the opposite pattern, which suggests cellular events regulated at a different level within a common signal transduction pathway.

Introduction
Acute promyelocytic leukaemia (APL) is characterised by the proliferation of leukaemic blast cells carrying the t(15;17) translocation, which fuses the promyelocytic gene on chromosome 15 to the retinoic acid receptor (RAR) α gene on chromosome 17 (1). APL also differs from other types of leukaemias by its association with bleeding diathesis processes (2). These processes include, among others, disseminated intravascular coagulation, as a result of increased tissue factor (TF) expression and reduced thrombomodulin (TM) expression by APL blast cells (2-4).

Therapy with all-trans-RA (ATRA) has become a major advance for the resolution of bleeding disorders in APL. ATRA causes differentiation of promyelocytic blast cells into mature myeloid cells (1) as well as down-regulation of procoagulant activities and up-regulation of TM expression (3, 5, 6). Granulocytic maturation also can be induced by the cAMP analogue dibutyryl cAMP (dbcAMP) (7). Transcription mechanisms underlying the action of ATRA have been recently described (8), but the signal transduction pathways associated with differentiation and modulation of TF and TM expression have not been fully established.

In some target cells, ATRA induces hydrolysis of inositol-containing phospholipids (9). This reaction is catalysed by phospholipase (PL) C and yields inositol triphosphate (IP₃) that mobilises Ca^{2+} and diacylglycerol (DAG), which in turn stimulates protein phosphorylation via protein kinase (PK) C activation (10). Inositide pathway activation has been classically associated with short-term cell responses (11), but it is not clear whether it plays a role in processes such as those associated with the promyelocytic cell differentiation, which involves long-term regulation of cell behaviour. In the present study, we analysed inositol incorporation during ATRA-induced granulocytic differentiation and the effects of inositol pathway inhibitors on CD11b, TF and TM expression. These studies were carried out in human NB4 cells, the unique promyelocytic cell line bearing the t(15; 17) translocation (12).

Haemopoietic cell differentiation is also associated with changes in protein phosphorylation, and different PKs and phosphatases are involved in these processes (13-16). PKC and PKA pathways have been proposed to mediate ATRA- and dbcAMP-induced differentiation of HL-60 cells (17), but there is no information about the role of PKs in TF and TM expression during promyelocytic cell differentiation. In the last years, with the knowledge of the molecular pathophysiology of some neoplastic processes associated with a consistent acquired genetic abnormality, several approaches have been conceived to develop innovative and efficient therapeutic strategies (18-20). With this in mind, we have attempted to clarify the molecular pathways involved in the response of promyelocytic cells to differentiating therapy by analysing the role of the PKC and PKA pathways in the regulation of CD11b, TF and TM expression.

Materials and Methods
Cells. Promyelocytic NB4 cells were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and cultured under conditions previously described by Duprez et al. (21). Cell viability was assessed by the trypan blue exclusion test.
Nitroblue tetrazolium (NBT) reduction assay. The degree of differentiation was assessed by the ability of cells to produce superoxide as measured by reduction of NBT as previously described by Collins et al. (22).

Flow cytometry analysis. Cells were washed in PBS, incubated for 30 min at 4°C with PBS containing 20% heat-inactivated normal human serum, washed again and incubated for 30 min at 4°C with 15 nM specific monoclonal antibodies to human TF (clone TP9-6B4, fluorescein isothiocyanate [FITC]-conjugated; American Diagnostica, Greenwich, CT), to human TM (unlabelled; American Diagnostica) or to CD11b (R-phycocerythrin [PE]-conjugated; Caltag Laboratories, San Francisco, CA). For analysis of cell surface TM, cells were subsequently washed twice in PBS containing 5% fetal bovine serum and incubated for 30 min at 4°C with goat anti-mouse IgG (FITC-conjugated; Sl Louis, MO) at a 1/100 final dilution. Flow cytometry analysis was performed on a FACScan (Becton Dickinson, San Jose, CA). Control cells incubated with FITC- or PE-conjugated non-specific antibodies (Becton Dickinson) were used to set the threshold for the fluorescence parameter such that the fraction of cells with positive fluorescence was < 2.5% of total cells. The percentage of TF (or TM, or CD11b) positive cells was determined from the fraction of cells in the sample incubated with specific antibodies that exceeded in fluorescence signal intensity the threshold obtained with the control sample.

Analysis of TF and TM mRNA accumulation. After culture NB4 cells were processed for RNA isolation as previously described (23). The Access reverse transcription and polymerase chain reaction (RT-PCR) System (Promega, Madison, WI) was used for the RT-PCR amplification of TF and TM mRNA from total cellular RNA. A reaction mixture was prepared as previously described (23) to get either 50 pM each of forward and reverse TF specific oligonucleotide primers (forward primer 5'-TCT CCC GTA ACC CAC TGG AA-3'). RT reaction to produce first-strand cDNA synthesis was initiated by adding 1 mU template RNA to the reaction mixture and was accomplished at 48°C for 45 min. PCR amplification was as previously described (23). Ten-μl aliquots of the total PCR products were analysed by ethidium bromide-stained agarose gel electrophoresis and visualised by UV transillumination using a Gel Documentation System (UVP Inc, Upland, CA). Positive images were produced on a black and white monitor screen as captured by the UVP video camera. The images were then saved to the MAC.TIF format to quantify TF and TM mRNA levels using image analysis software (GelBase/GelBlot Pro, UVP Inc). Results were calculated in terms of integrated optical density and expressed in arbitrary units.

Assay of inositol uptake. Cell culture medium was supplemented with 3 μCi/ml [3H]-myo-inositol (Amersham, Buckinghamshire, UK) to determine its incorporation into NB4 cells. After incubation, cells were washed twice in Hepses-inositol buffer (10 mM Hepes [Sigma] pH 7.4, 135 mM NaCl, 4.5 mM KCl, 0.5 mM MgCl2 × H2O, 1.5 mM CaCl2, 2 mg/ml BSA [Sigma], 1 mg/ml glucose and 1 mM inositol [Sigma]). Adding 50 μl of ice-cold 20% trichloroacetic acid stopped [3H]-myo-inositol incorporation. Cells were then sonicated and centrifuged (12900 × g for 15 min at 4°C). Assays were performed as previously described by Bijsterbosch and Klaus (24). Results are reported as the total amount of [3H]-myo-inositol incorporated in each sample (IPs plus phosphoinositides) and expressed as cpm per mg total protein.

Assay of cAMP accumulation. After culture NB4 cells were washed in 0.01 M PBS containing 4 mM EDTA to prevent enzymatic degradation of cAMP. Samples were subsequently sonicated for 5 min and warmed in boiling water to coagulate protein. Aliquots for protein determination were taken from each well. After centrifugation (8000 × g for 5 min), supernatants were removed and stored at −20°C until cAMP assay. cAMP was quantified using a commercial cAMP [3H] assay system (Amersham). Results are reported as pmol of cAMP per mg total protein.

Analysis of PKC and PKA activity. PKC and PKA activity in NB4 cells were quantified using commercial enzyme immunoassays (Calbiochem-Novabiochem, La Jolla, CA) following manufacturer’s specifications. PK activity was determined by reference to a standard curve generated with serial dilutions of commercial PK, either purified mouse brain PKC (Calbiochem-Novabiochem) or the recombinant catalytic subunit of mouse brain PKA (Calbiochem-Novabiochem). Results were expressed as μU of PKC or PKA activity per mg total protein. One unit of PKC activity is defined as the amount of enzyme that will transfer 1.0 nmol of phosphate to histone III-S per min at 22°C, pH 7.4. One unit of PKA activity is defined as the amount of enzyme that will transfer 0.1 nmol of phosphate to kemptide (synthetic substrate for PKA [H-Leu-Arg-Arg-Ala-Ser-Leu-Gly-0H, Km =16 μM]) per min at 30°C, pH 7.5.

Table 1 Effects of PK inhibitors on the modulation of CD11b, TF and TM expression on NB4 cells treated with ATRA or dbcAMP

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Inhibitor</th>
<th>CD11b FACS (%+ cells)</th>
<th>mRNA (AU ofIOD)</th>
<th>TF FACS (%+ cells)</th>
<th>mRNA (AU ofIOD)</th>
<th>TM FACS (%+ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>5.7 ± 2.6</td>
<td>5.3 ± 0.2</td>
<td>98.6 ± 1.2</td>
<td>1.0 ± 0.0</td>
<td>7.7 ± 2.3</td>
</tr>
<tr>
<td>None</td>
<td>Phloretin</td>
<td>4.7 ± 0.6</td>
<td>5.1 ± 0.1</td>
<td>95.5 ± 2.5</td>
<td>1.3 ± 0.2</td>
<td>6.5 ± 1.4</td>
</tr>
<tr>
<td>None</td>
<td>PKC[19-31]</td>
<td>6.0 ± 1.2</td>
<td>4.9 ± 0.3</td>
<td>90.4 ± 1.3</td>
<td>1.3 ± 0.2</td>
<td>7.9 ± 0.9</td>
</tr>
<tr>
<td>None</td>
<td>H8</td>
<td>5.4 ± 0.9</td>
<td>4.2 ± 0.2</td>
<td>99.1 ± 2.8</td>
<td>1.1 ± 0.2</td>
<td>8.2 ± 2.6</td>
</tr>
<tr>
<td>ATRA</td>
<td>None</td>
<td>92.6 ± 5.6</td>
<td>0.9 ± 0.0</td>
<td>12.5 ± 1.9</td>
<td>3.5 ± 0.2</td>
<td>88.8 ± 3.5</td>
</tr>
<tr>
<td>ATRA</td>
<td>Phloretin</td>
<td>19.3 ± 2.5</td>
<td>1.8 ± 0.1 ab</td>
<td>27.6 ± 0.9 ab</td>
<td>2.9 ± 0.0 ab</td>
<td>59.4 ± 2.5 ab</td>
</tr>
<tr>
<td>ATRA</td>
<td>PKC[19-31]</td>
<td>56.9 ± 4.8</td>
<td>2.2 ± 0.1 abc</td>
<td>40.9 ± 1.7 abc</td>
<td>1.6 ± 0.1 abc</td>
<td>42.2 ± 1.8 abc</td>
</tr>
<tr>
<td>ATRA</td>
<td>H8</td>
<td>90.4 ± 1.7</td>
<td>1.3 ± 0.1 a</td>
<td>13.1 ± 0.9 a</td>
<td>3.7 ± 0.0 a</td>
<td>79.2 ± 1.6 a</td>
</tr>
<tr>
<td>dbcAMP</td>
<td>None</td>
<td>59.7 ± 5.5</td>
<td>2.5 ± 0.1 a</td>
<td>30.7 ± 4.4 a</td>
<td>2.9 ± 0.2 a</td>
<td>45.2 ± 4.6 a</td>
</tr>
<tr>
<td>dbcAMP</td>
<td>Phloretin</td>
<td>69.2 ± 5.1</td>
<td>3.0 ± 0.3 a</td>
<td>41.6 ± 3.9 a</td>
<td>2.5 ± 0.3 a</td>
<td>41.2 ± 6.2 a</td>
</tr>
<tr>
<td>dbcAMP</td>
<td>PKC[19-31]</td>
<td>57.5 ± 2.5</td>
<td>2.9 ± 0.0 a</td>
<td>32.0 ± 1.3 a</td>
<td>2.6 ± 0.1 a</td>
<td>47.4 ± 3.6 a</td>
</tr>
<tr>
<td>dbcAMP</td>
<td>H8</td>
<td>81.2 ± 2.8</td>
<td>5.0 ± 0.3 b</td>
<td>64.2 ± 4.8 b</td>
<td>1.2 ± 0.0 b</td>
<td>23.5 ± 1.9 b</td>
</tr>
</tbody>
</table>

NB4 cells were incubated with 1μM ATRA or 250 μM dbcAMP for 48 h. The contribution of IP, PKC and PKA in the expression of CD11b, TF and TM during induced differentiation was studied by the use of: phloretin (20μM) as inositol uptake and PKC inhibitor, PKC[19-31] as specific PKC inhibitor at 100 nM, and H8 as specific PKA inhibitor at 10μM. CD11b, TF and TM cell surface expression was analysed in intact cells by FACS using specific monoclonal antibodies. Results are expressed as percentage of positive cells (see details in text). TF and TM mRNA were amplified by RT-PCR and results were calculated as described in Materials and Methods and expressed as arbitrary units (AU) of integrated optical density (IOD). Results are the mean ± the standard error of mean of three separate experiments. P < 0.05, a vs cells incubated with medium only; b, vs the cells incubated with the inducer alone; c, vs the cells incubated with ATRA plus phloretin.
Reagents and stock solutions. ATRA, dbcAMP and the specific PKA inhibitor H8 (25, 26) were obtained from Sigma. The specific PLC inhibitor U73122 (27) was from RBI (San Diego, CA). The myo-inositol uptake and PKC inhibitor phloretin (28, 29) and the specific PKC inhibitor peptide PKC[19-31] (30) were from Calbiochem-Novabiochem. The stock solutions were as follows: ATRA (100 mM) and phloretin (3.6 mM) in ethanol; dbcAMP and H8 (1 mM each) in distilled water; U73122 (1 mM) in dimethyl sulfoxide; and PKC[19-31] (13 μM) in 5% acetic acid. Stock solutions were stored at −20°C. For each experiment, the stock solutions were diluted in culture medium to obtain the required concentration of the substance of interest. The inducers or inhibitors used were dissolved in ethanol, dimethyl sulfoxide or acetic acid such that the final concentration of solvent (0.1%) did not affect the growth or differentiation of the cells. In addition, control cells received the same calculated volume of such diluents.

Statistical analysis. Results were expressed as mean ± the standard error of mean. Statistical analysis was carried out with the SigmaStat software package (Jandel Scientific, Corte Madera, CA). Statistical differences were assessed using Anova. Differences were considered significant if P < 0.05.

Results

Effect of PK inhibitors on the expression of TF and TM during the differentiation of NB4 promyelocytic cells. NB4 can be induced to granulocytic differentiation by treatment with either ATRA or dbcAMP (1, 7). To explore the participation of PKC and PKA pathways during these processes we treated NB4 cells with ATRA and dbcAMP respectively, in the presence or absence of PKC and PKA pathway inhibitors and the induced granulocytic differentiation as well as the modulation of TF and TM expression was analysed. As summarised in the Table 1, both ATRA and dbcAMP induced differentiation of NB4 cells to a granulocytic phenotype as assessed by CD11b expression on cell surfaces analysed by flow cytometry. Differentiation also was confirmed by NBT assays: 78% and 56% of ATRA- and dbcAMP-treated cells, respectively, were NBT positive compared to <6% of untreated cells. Granulocytic differentiation induced by ATRA was blocked by inhibition of the IP/PKC pathway, the intensity of changes being dependent on the inhibitor used. For example, phloretin was more effective in reversing the effects of ATRA on CD11b expression than PKC[19-31], while the PKA inhibitor H8 did not change the pattern of CD11b expression. In contrast, H8 markedly reduced CD11b expression induced by dbcAMP, but this was not affected by the addition of phloretin or PKC[19-31]. These results were also confirmed by analysis of the NBT-reducing ability of NB4 cells. Combined treatment of ATRA and PKC[19-31] or phloretin inhibited the ATRA-induced differentiation by 40% and 75% respectively, while H8 reduced by 70% the differentiation promoted by dbcAMP. These data suggested that the IP/PKC pathway mainly mediated ATRA-induced differentiation of NB4 cells, whereas the PKA pathway mediated the differentiation induced by dbcAMP.

With this approach we also analysed the expression pattern of TF and TM on cells treated with either ATRA or dbcAMP in the presence or absence of PK inhibitors. TF and TM expression were explored by RT-PCR amplification of their mRNA and by flow cytometry analysis with specific monoclonal antibodies against these receptors. As summarised in the Table 1, the down-modulation of TF expression and the up-modulation of TM induced by ATRA were blocked by IP/PKC inhibitors, PKC[19-31] being more effective than phloretin while H8 did not affect these changes. Conversely, only the PKA inhibitor H8 abrogated the modulation of these receptors by dbcAMP, with no changes induced by the addition of PKC inhibitors.

Inositol incorporation and PK activities during differentiation of NB4 cells induced by ATRA and dbcAMP treatment. With the evidence that PKC and PKA pathways mediated the effects of ATRA and dbcAMP on NB4 cells, respectively, we further explored the mechanisms underlying these effects. First, to analyse whether treatment of cells with ATRA or dbcAMP modified cellular inositol uptake, NB4 cells were treated with these inducers for 48 h and the incorporation of [3H]-myo-inositol was measured. As shown in Fig. 1, ATRA treatment of cells induced a 57% decrease in [3H]-myo-inositol incorporation in NB4 cells. Two mg of logarithmically-growing NB4 cells were plated in 12-well cell culture plates at a density of 2.5 × 10<sup>5</sup> cells/ml in medium alone, medium containing 1 μM ATRA or medium containing 250 μM dbcAMP. Cells were cultured for 48 h without inositol uptake inhibitors or in the presence of 20 μM phloretin or 5 μM U73122. Myo-inositol incorporation was measured as described in Materials and Methods. Results are the mean of three independent experiments ± the standard error of mean. Differences at P < 0.05: a. vs. the cells incubated without inducer (control); b. vs. cells incubated with 1 μM ATRA only
Second, we measured PKC and PKA activities following treatment of NB4 cells with all-trans-retinoic acid (ATRA) or dibutyryl cAMP (dbcAMP). Cells were plated in medium alone, medium containing 1 μM ATRA or medium containing 250 μM dbcAMP as described in the legend to Fig. 1, and cultured for 24 h without PK inhibitors or in the presence of 20 μM phloretin, 100 nM PKC[19-31] or 10 μM H8. PKC activity (panel A) and PKA activity (panel B) were measured as described in Materials and Methods. Inset of panel B shows the time course of intracellular cAMP generation in NB4 cells incubated with medium only, ATRA or dbcAMP. Results are the mean of four independent experiments ± the standard error of mean. Differences at P < 0.05: a, vs. cells incubated with medium only; b, vs. the cells incubated with the inducer alone; c, vs. the cells incubated with ATRA plus PKC [19-31].

Intracellular signalling of NB4 cells induced to granulocytic differentiation with dbcAMP was quite different from that induced by ATRA. Under treatment with dbcAMP no change in [3H]-myo-inositol uptake and PKC activity was observed but both intracellular cAMP and PKA activity were increased (4.0 fold and 2.6 fold, respectively, over the control values at 24 h, Fig. 2), while no changes in cAMP or in PKA activity were observed after ATRA treatment. In addition, only the PKA inhibitor H8 inhibited dbcAMP-induced PKA activity and reversed the effects of dbcAMP on the expression of CD11b, TF and TM (Table and Fig. 2) while H8 did not affect the expression of these cellular receptors induced by ATRA. Therefore, we propose that the effects of ATRA on TF and TM expression are more closely related to PKC activity than to inositol uptake and thus, could be regulated at a different level within the same signal transduction pathway. These results confirm and delineate mechanisms of previously described observations suggesting that ATRA down-regulates TF expression on NB4 cells independently of cell differentiation (31).

Intracellular signalling of NB4 cells treated with ATRA or dbcAMP was quite different from that induced by ATRA. Under treatment with dbcAMP no change in [3H]-myo-inositol uptake and PKC activity was observed but both intracellular cAMP and PKA activity were increased (4.0 fold and 2.6 fold, respectively, over the control values at 24 h, Fig. 2), while no changes in cAMP or in PKA activity were observed after ATRA treatment. In addition, only the PKA inhibitor H8 inhibited dbcAMP-induced PKA activity and reversed the effects of dbcAMP on the expression of CD11b, TF and TM (Table and Fig. 2) while H8 did not affect the expression of these cellular receptors induced by ATRA. Therefore, we propose that the effects of ATRA on TF and TM expression are more closely related to PKC activity than to inositol uptake and thus, could be regulated at a different level within the same signal transduction pathway. These results confirm and delineate mechanisms of previously described observations suggesting that ATRA down-regulates TF expression on NB4 cells independently of cell differentiation (31).

**Discussion**

Haemopoietic cell differentiation is associated with changes affecting different signal transduction pathways (9, 10, 15). In the present study we have analysed the phenotypic changes induced in NB4 cells by ATRA and dbcAMP in the presence of different IP/PKC and PKA inhibitors to delineate mechanisms underlying the regulation of TF and TM expression in differentiating promyelocytic cells. Phenotypic changes were more deeply explored by analysing myo-inositol incorporation, intracellular cAMP generation and PKC and PKA activities during treatment of NB4 cells with ATRA and dbcAMP. The use of several inhibitors in these studies allowed us to explore the distinct participation of components of IP/PKC and PKA pathways in these effects.

With these approaches, we found that on ATRA-treated NB4 cells, differentiation and changes in TF and TM expression were significantly abrogated when PKC pathway inhibitors, phloretin and PKC[19-31], were added to cells. Conversely, the PKA pathway inhibitor H8 blocked differentiation and TF and TM modulation induced by dbcAMP. However, differences in the effect of PK inhibitors used were found with respect to granulocytic differentiation and TF and TM modulation. For example, on ATRA-treated cells, phloretin was more effective in reversing the effects of ATRA on differentiation than PKC[19-31]. As phloretin apart from inhibiting PKC activity also blocks myo-inositol uptake, we propose that both myo-inositol uptake and PKC activity mediate differentiation induced by ATRA. In contrast, PKC[19-31], a specific inhibitor of PKC activity at the concentration used in our studies, was more effective than phloretin in reversing the effects of ATRA on TM and TF expression, suggesting that modulation of TF and TM was mainly mediated by PKC activity. These data indicate that the modulation of TF and TM are more closely related to PKC activity than to inositol uptake and thus, could be regulated at a different level within the same signal transduction pathway. These results confirm and delineate mechanisms of previously described observations suggesting that ATRA down-regulates TF expression on NB4 cells independently of cell differentiation (31).
volved in their differentiation. Differences between HL-60 and NB4 cells also have been previously described with respect to the modulation of nuclear receptor expression induced by ATRA or cAMP analogues: ie, in NB4 cells cAMP and ATRA have opposite effects on retinoid-X-receptor (RXR) gene regulation, as well as on the expression at protein level of both the RARα and the RXRα (33), indicating that these cell lines, bearing distinct translocations, are not comparable.

Taken together, our results indicate that: 1) the differentiation and TF and TM modulation of ATRA-treated NB4 cells is mediated by the IP/PKC pathway, 2) differentiation of ATRA-treated cells is mainly mediated by inositol uptake while TF and TM modulation are mainly mediated by PKC activation, 3) NB4 cells can also be induced to granulocytic differentiation and TF and TM modulation by the independent activation of PKA pathway with similar anticoagulant effects to those induced by ATRA, and 4) the IP/PLC/PKC pathway plays a major role in mediating the global anticoagulant effects of retinoids on promyelocytic cells. The ongoing search for proteins and pathways associated with the abnormal phenotype of the APL cells will surely uncover new molecular targets for a rational therapy of APL in the near future.

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