Retinoic acid—A player that rules the game of life and death in neutrophils

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Neutrophils are the most prevalent white blood cells in the circulation. They represent the first line of defense against invading microorganisms and have been implicated in the pathogenesis of a number of human diseases. In response to various factors, the pluripotent stem cells in bone marrow differentiate into mature neutrophils, enter the blood stream, and die within 24 hr via apoptosis. Numerous defects can occur during the process of neutrophils' differentiation that can manifest in the form of a variety of clinical disorders. Retinoids (Vitamin A and analogues), in general, and all-trans retinoic acid (tRA), in particular, play a critical role during differentiation of neutrophils. tRA can directly modulate gene expression via binding to its nuclear receptors, which in turn, can activate transcription of genes that are essential for differentiation of immature cells to neutrophils. Involvement of retinoic acid receptor in pathogenesis of acute promyelocytic leukemia (APL), reflects an important role played by this receptor in differentiation of immature myeloid cells to neutrophils. This review summarizes evidence on involvement of retinoic acid-mediated events in differentiation process of neutrophils and their subsequent apoptosis.

Neutrophils play a major role in host defense mechanisms against microorganisms and in acute inflammation. On an average, about ten million neutrophils are produced and released every minute in the blood stream of an adult human being. However, the net number remains constant, suggesting that an equal number of neutrophils must be deleted constantly in order to maintain normal homeostasis. A series of events that are orchestrated by a variety of factors in the bone marrow lead to the differentiation of primitive hematopoietic precursor stem cells into terminally differentiated neutrophils. Once mature, they enter the blood circulation or the tissues where they function as effector cells in host-defense mechanisms and after relatively short periods they are eliminated from the body through a genetically regulated mechanism called apoptosis, or programmed cell death. A critical balance between life span of these cells and their regulated death is important for normal homeostasis.

Certain molecular flaws in differentiation process can manifest in the form of clinical disorders. The observations concerning these disorders have led to understanding the basic mechanisms involved in myelopoiesis. Particularly, studies of acute promyelocytic leukemia (APL), a subtype of acute myeloid leukemia that is caused by a block in differentiation at the promyelocytic stage, have revealed a crucial role for tRA in the process of neutrophil differentiation. Similarly, the human myeloblast leukemia cell line, HL-60, has provided an excellent in vitro model for studying the regulatory mechanisms of myeloid cell differentiation. This cell line can be induced with tRA to differentiate toward cells sharing several phenotypes of mature neutrophils. tRA-differentiated HL-60 cells, like normal neutrophils, have a limited in vitro life span and undergo spontaneous apoptosis. Thus, HL-60 cells are a good model, not only to investigate the molecular events linked with neutrophils differentiation, but also to study the apoptotic processes involved in programmed cell death. An important feature of neutrophil physiology is their rapid and spontaneous programming for cell death, a major mechanism that maintains neutrophil homeostasis in vivo. It is well-known that apoptosis play an important role in clearing neutrophils from inflamed tissue, which is critical for limiting the injury to inflamed tissue and subsequent resolution of inflammation. However, the molecular mechanisms controlling neutrophils apoptosis remain largely unknown. This review summarizes the current information on molecular events that lead to tRA-induced differentiation and apoptosis in myeloid cells.
**tRA as transcription regulators**

As pointed out earlier, tRA plays an important role in neutrophil differentiation pathway by regulating the cell growth, differentiation and cell death functions. The ability of retinoids to influence these complex biological processes resides in their ability to modulate the expression of multiple genes (Table 1). Some of these genes are expressed immediately following treatment with tRA, while the expression of other genes is delayed. Net effect of tRA-induced gene expression is the inhibition of cell growth and induction of differentiation and apoptosis. tRA-induced expression of target genes is mediated through nuclear receptors that belong to a superfamily of ligand-inducible transcription factors including steroid, vitamin D, thyroid hormone, and peroxisome proliferator-activated receptor. Specifically, retinoids can bind and activate two major types of nuclear receptors, the retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Both, RARs and RXRs consist of three subtypes (α-, β- and γ-) that are encoded by separate genes and have been discussed in greater detail in several recent reviews.

Each of RAR and RXR subtypes contains six distinct domains referred to as, A through F, and each of these domains has diverse functions (Fig. 1A). A and B domains are located at the amino terminal and contain isoform specific, ligand-independent transactivation functions, AF-1. The DNA-binding domain (domain C) is highly conserved among different retinoid receptor isoforms and binds to specific upstream nucleotide sequences (referred to as retinoic acid response element or RARE), located in the promoter region of the target genes. (Fig. 1B). Ligands (retinoids) bind to the ligand-binding domain (LBD) or E domain at C-terminus of the receptors that contain sequences involved in dimerization of the receptors, ligand dependent transactivation (AF-2), and translocation to the nucleus. Functions for D and F domains have not been clearly defined. RARs and RXRs mainly act as heterodimers to affect gene transcription after binding to RAREs in the promoter region of target genes (Fig. 1B). RARs can be transcriptionally activated by binding to either tRA or 9-cis-RA, however RXRs can be activated only by 9-cis-RA (Fig. 1C). Upon ligand binding, activated nuclear receptors that bind to RAREs found in the upstream sequences (promoters) of RA responsive genes and lead to transactivation of the target genes.

Complex diversity and pleiotropic effects in tRA signaling pathway is provided not only due to existence of multiple isoforms of RA receptors but also as

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**Fig. 1** — Structure and functions of retinoid receptors. (A) — Schematic representation of retinoid receptor protein depicting various functional domains. (B) — A molecular model for retinoid action. The liganded RAR forms heterodimer with RXR, binds to specific regulatory sequences (RARE) in the promoter region of a target gene A. Transactivation of gene A is a primary event of retinoid action. In an another scenario, the product of gene A can itself activate the transcription of a second gene B. Transactivation of gene B represents a secondary action of retinoid since its transcription requires the synthesis of protein A. This cascade of gene events leads to secondary and tertiary events that eventually produce a phenotype that is characteristic of retinoid action. (C) — Natural isoforms of retinoid receptors (RARs and RXRs) and their physiological ligands are listed.
a result of combinations of RAR-RXR heterodimers and presence of different ligands. tRA treatment, for example, can induce expression of RARα and RARβ genes, suggesting that tRA can modulate its own receptors, in addition to differentiation-related genes. Availability of retinoid ligands to its cognate receptors can be regulated by the level of certain non-receptor proteins, such as cytoplasmic retinoic acid binding proteins (CRABP) and heat shock proteins. Formation of RXR/RAR heterodimer is required for high-affinity binding to RAREs that is critical for subsequent retinoid-induced transcription of target genes. Most RAREs have been identified in the regulatory regions of genes whose transcription is induced by retinoids (Table 1). These cognate response elements consist of direct repeats (DR) of 2 core motifs in the sequence AGGTCA(X)nAGGTCA. The most common spacing observed in RAREs is 5 bp (DR5); however, RAREs containing these or similar motifs separated by 1 (DR1) or 2 bp (DR2) are also common.

Current view on tRA-mediated regulation of target gene expression is that the unliganded RAR-RXR heterodimer constitutes a high affinity receptor and can bind to the RARE in upstream sequence of the target gene. Nevertheless, in the absence of ligand or in the presence of some antagonists, the receptor’s target gene stays in repressed form. This is due to the recruitment of histone deacetylase (HDAC) containing complexes that are tethered through corepressors, such as the silencing mediator for retinoid and thyroid hormone receptors (SMRT) or the nuclear receptor co-repressor (NCoR) to a non-ligated RAR-RXR dimer (Fig. 2A). This process results in histone deacetylation, chromatin compaction and silencing of the target-gene. Current understanding of RAR-mediated gene transcription is illustrated in Figure 2B. The binding of RAR ligand (e.g. tRA) destabilizes the corepressor binding and induces allosteric changes in the ligand-binding domain. This results in the release of repressor-protein complex and subsequent recruitment of transcriptional coactivator complexes (containing CREB-binding protein [CBP]/p300 or other co-activators) that contain or recruit histone deacetylases.

Table 1 — Modulation of gene expression by tRA during neutrophil differentiation

<table>
<thead>
<tr>
<th>Target gene</th>
<th>RARE in the promoter</th>
<th>Function</th>
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<tbody>
<tr>
<td>CD38</td>
<td>Yes</td>
<td>Differentiation</td>
</tr>
<tr>
<td>C/EBPα</td>
<td>Yes</td>
<td>Differentiation</td>
</tr>
<tr>
<td>CIP/WAF/p21</td>
<td>Yes</td>
<td>Cell cycle inhibitor</td>
</tr>
<tr>
<td>Transglutaminase</td>
<td>Yes</td>
<td>Adhesion</td>
</tr>
<tr>
<td>CD11b</td>
<td>Yes</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>Myeloperoxidase</td>
<td>Yes</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>TRAIL</td>
<td>?</td>
<td>Pro-inflammatory</td>
</tr>
<tr>
<td>PU.1</td>
<td>Yes</td>
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</tr>
<tr>
<td>RARβ</td>
<td>Yes</td>
<td>Differentiation</td>
</tr>
<tr>
<td>STATs</td>
<td>Yes</td>
<td>Transcription factors</td>
</tr>
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<table>
<thead>
<tr>
<th>Downregulated</th>
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<tbody>
<tr>
<td>e-my c</td>
<td>?</td>
<td>Cell growth</td>
</tr>
<tr>
<td>bcl2</td>
<td>?</td>
<td>Apoptosis</td>
</tr>
</tbody>
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Fig. 2 — Schematic representation of the basal repression and retinoid-induced transcriptional regulation of the target gene(s).
(A)—Nucleosomes consist of DNA (black line) wrapped around histone octamers (black circles). The binding of unligated RAR/RXR heterodimer to RARE (DR5-RARE) in promoter region of the target gene, results in the recruitment of a transcriptional repressor complex that comprises N-CoR, mSm3a, SMRT, and a histone deacetylase (HDAC-1). HDAC-1 activity helps to suppress the transcriptional activity. (B)—Addition of tRA results in a conformational change in RAR/RXR heterodimer that leads to release of repressor complex and recruitment of a transcriptional-activator complex that has histone acetyltransferase (HAT) activity. Histone acetylation (Ac) of DNA relaxes chromatin and creates a permissive state for promoter activation.
acetyltransferase (HAT)\textsuperscript{12,14}. Thus, binding of ligand to RAR/RXR dimers converts it from a transcriptional repressor (Fig. 2A) to a transcriptional activator (Fig. 2B). Evidently, modulation of transcriptional activity involves close links between acetylation/deacetylation and stabilization/destabilization of repressive chromosomes.

Retinoid receptors in neutrophil differentiation and apoptosis

In hematopoietic cells, apoptosis appears to be an essential component of terminal differentiation. Under steady-state conditions, large production of neutrophils is counterbalanced by their elimination in the tissues without eliciting an inflammatory response. Aging neutrophils typically die by apoptosis and are subsequently cleared by local macrophages. Many cytokines, such as GM-CSF, G-CSF, interleukin-3, and interleukin-5, can increase the circulating time of mature granulocytes from 8 to 48 hr, probably by delaying their apoptosis\textsuperscript{15}. Despite many such observations, little information is available on the biological signals that trigger the process of apoptosis in normal neutrophils.

It is apparent that retinoid-induced differentiation and apoptosis in myeloid cells are receptor-mediated and receptor-selective events. Using human myeloid leukemia (HL-60) cells that expressed specific subtypes of retinoid receptor (RAR or RXR), we demonstrated that activation of RAR\textalpha was sufficient to induce terminal differentiation of HL-60 cells into neutrophils, while ligand activation of RXR\textalpha induced DNA fragmentation and morphological changes, characteristic of apoptosis\textsuperscript{16}. Similarly, Nagy \textit{et al.}\textsuperscript{17} have used receptor-specific synthetic retinoids that preferentially activate either RARs or RXRs to demonstrate that ligand activation of RARs is necessary to induce differentiation, whereas activation of RXRs is essential for the induction of apoptosis in HL-60 cells. Yet in another study, Ueno \textit{et al.}\textsuperscript{18}, by using a RAR-selective antagonist that prevents retinoid-induced RARs-dependent transactivation of target genes, have obtained similar results. One major caveat of using synthetic retinoids in these studies is that these compounds can be metabolized or isomerized inside the cell and thus may exhibit altered receptor specificity. In contrast, we have utilized retroviral vector-mediated gene transduction to introduce RAR\textalpha or RXR\textalpha genes into HL-60R cells, which harbor a mutated non-functional RAR\textalpha gene\textsuperscript{19}. We have observed that 9-cis RA treatment of RXR\textalpha transduced cells primarily lead to apoptosis of HL-60 cells, while tRA

![Diagram](image)

**Fig. 3** — Retinoid-induced differentiation and apoptosis of myeloid leukemia HL-60 cells. (A) — HL-60R cells harbour a functional mutation in RAR\textalpha gene that results in a non-functional RAR\textalpha protein and renders the cells resistant to tRA. (B) — Retrovirally-transduced expression of functional RAR\textalpha in HL-60R cells restores the ability of these cells to differentiate in response to tRA treatment. (C) — Transduction of RXR\textalpha cDNA, renders HL-60R cells highly sensitive to RXR-selective ligands (e.g., 9-cis RA) and induce massive apoptosis in these cells without morphological differentiation. (D) — RAR\textalpha nuclear receptors in wild-type HL-60 cells when bind to and are activated by appropriate ligand (e.g., tRA) result in granulocytic differentiation of the cells. The differentiation process is associated with induction of several new genes, including RXR\textalpha receptors. It is conceivable that tRA is isomerized to 9-cis RA \textit{in situ}, which can then bind and activate RXRs, leading to onset of apoptosis in differentiated HL-60 cells.
treatment of RARα-transduced cells primarily induces granulocytic differentiation (Fig. 3)\(^6\). These studies, using different experimental approaches, reach similar conclusions and support the involvement of RAR and RXR in the regulation of differentiation and apoptosis, respectively, in HL-60 cells.

**APL: An in vivo model for tRA-induced differentiation**—During early 1990s, the elucidation of molecular pathways that lead to development of acute promyelocytic leukemia (APL) provided a strong paradigm for the connection between in vitro and in vivo observations and supported the role of tRA in committing hematopoietic cells to granulocytic pathway. APL is a subtype of acute myeloid leukemia that is characterized by a block in differentiation at the promyelocytic stage\(^6\). It is becoming increasingly evident that APL is uniquely sensitive to tRA and clinical trials indicate that tRA induces complete remissions by differentiation and eventual elimination of the malignant clone. The great majority (\(>99\%\)) of APLs exhibit a characteristic t(15;17) chromosome translocation that generates an aberrant chimeric protein (PML-RAR), fusing a portion of PML gene on chromosome 15 with RARα on chromosome 17 (Fig. 4). Several functional domains are altered in PML-RAR fusion. RARα is truncated at N-terminus and lacks A region (Fig. 4) which is considered to be essential for promoter transactivation. Other domains, including DNA-binding domain, ligand-binding domain, RXR heterodimerization domain are intact in PML-RXR. PML gene, on the other hand, is truncated at C-terminus resulting in loss of a serine rich region. This region is considered to be a target for phosphorylation.

PML-RAR fusion protein interferes with the physiologic functions of PML and RARα proteins and exerts dominant negative effect. Consequently, expression of PML-RAR fusion protein in APL cells blocks their differentiation at promyelocytic stage, leading to accumulation of immature hematopoietic cells in bone marrow. In agreement to this conviction, overexpression of a dominant negative RARα has been shown to cause a differential block at the promyelocytic stage\(^20\). Recently, transgenic mice expressing PML-RARα have block at the promyelocytic stage of myeloid maturation in blast cells implicating important role for PML-RARα, abnormal receptor protein in leukemogenesis\(^21,22\).

Moreover, PML protein is involved in regulation of cell proliferation and apoptosis. Cells lacking PML

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**Fig. 4**—Expression of oncogenic RARα receptor protein in APL cells due to chromosomal translocation. Chromosomal translocation involves RARα gene on chromosome 17 and promyelocytic leukemia (PML) gene on chromosome 15. The breakpoints may vary in PML gene; however, it is always located in the same point in RARα gene. tRA at physiologic concentrations (1–10 nM) can bind and activate wild-type RARα, but not PML-RARα. As a result of this, APL cells, which harbour PML-RARα fusion protein are blocked in promyelocytic stage of differentiation. However, at pharmacological concentrations (\(>1 \mu M\)), tRA can activate the fusion protein, causing transactivation of target genes, differentiation of APL cells and remissions in APL patients.
are resistant to apoptosis by gamma irradiation, grow faster and have longer survival time, while cells over expressing PML undergo apoptosis by the same stimulus\(^2\). It has been shown that PML is located in the nucleus of normal cells in punctated structures (PODs) or nuclear bodies associated with nuclear matrix, however, in PML-RAR\(\alpha\) positive APL cells, localization and the normal pattern of nuclear bodies are disrupted. Overall, the data obtained suggest that disruption of PML function may also contribute to APL pathogenesis.

Based for dramatic efficacy of tRA against APL is the ability of pharmacological doses of tRA to overcome repression of signaling caused by PML-RAR fusion protein. Restoration of signaling leads to differentiation of APL cells and then to postmaturation apoptosis\(^23\). tRA induces differentiation of immature leukemic blasts into terminally differentiated granulocytic cells that is associated with clinical remissions (Fig. 4).

**tRA-inducible genes in neutrophil differentiation and apoptosis**

Modulation of several putative target genes during tRA-induced maturation of neutrophils suggests that tRA can directly affect neutrophil maturation by switching on or off certain subset of genes. These genes include early response genes (ERGs) that possess functional RARE within their promoter and can be induced in the presence of cyclohexamide. Induction of ERGs require tRA-induced heterodimerization of RAR-RXR which can then bind and activate the target promoter in conjunction with other transcriptional factors. Secondary response genes include those genes that exhibit tRA induction but fail to be expressed in the presence of cyclohexamide and do not possess RARE within their promoters (Fig. 1B).

Several members of C/EBP family of transcription factors have been shown to play a role in hematopoiesis. C/EBP\(\alpha\) in particular, has been implicated to be a tRA target gene during neutrophils maturation. For example, differentiation of APL cell line (NB4) is associated with induction of this gene as early as 1 hr after 9-cis RA treatment. Consistent with these findings was the observation that C/EBP\(\alpha\) promoter contains a functional RARE\(^24\). Similarly, CD38 that has been originally described as a differentiation marker on T and B cells, is rapidly induced in response to tRA treatment of HL-60 cells and APL cells and its promoter has a functional RARE\(^25\). The precise function of CD38 in neutrophils differentiation remains to be elucidated but it is known to catalyze the synthesis of cyclic ADP-ribose, a metabolite of NAD\(^+\) that can induce calcium mobilization\(^26\). CIP/WAF/p21 is another direct target gene of tRA whose promoter contains a functional RARE. The p21 protein binds and inhibits the function of a cyclin-dependent protein kinase resulting in growth arrest of the cells in G1 phase.

In contrast to C/EBP and p21 genes, the expression of c-myc oncogene is down-regulated by tRA in a cyclohexamide independent fashion\(^27\). In fact, the activation mediated by RAR\(\alpha\) is reminiscent of that exerted by Myc/Mad system in cell's decision to proliferate or differentiate. Several other genes have been identified whose expression is modulated by tRA in various myeloid cells. For example, recently Liu et al.\(^28\) have used the combined approaches of suppression-subtraction hybridization, differential display, and microarray to identify several genes that are induced in response to tRA treatment of APL cells. In another study, Lian et al.\(^29\) used the microarray and 2-D protein electrophoresis analysis to identify several known and new genes whose expression is modulated during tRA-induced differentiation of a murine promyelocytic leukemia cell line. Interestingly, in both the studies a large number of genes that are modulated by tRA, encoded proteins that are involved in cell growth and apoptotic regulatory pathways. Similar studies on the identification of critical genes that are regulated by unliganded and liganded RARs will be useful to understand the process of granulopoiesis at molecular level.

As discussed in a previous section, tRA-induced differentiation of neutrophils is shortlived and the differentiated cells are destined to undergo apoptosis within 24 hr. tRA can induce several changes that may render these cells susceptible to apoptosis. Increased expression of a protein crosslinking enzyme, tissue transglutaminase (TGase), for example, has been linked to retinoid-induced terminal differentiation and arrest of cell growth in several hematopoietic cells\(^30\). High levels of TGase have also been demonstrated in several cell types undergoing apoptotic cell death\(^31\). Activation of TGase is thought to be responsible for formation of intracellular cross-linked protein polymers that constitute the major component in an apoptotic envelope.

Does tRA-induced TGase expression has any role in the execution of apoptosis? Transfection studies have been carried out to directly address this question.
Introduction of full-length TGase cDNA in fibroblasts, human neuroblastoma, and L-929 fibroblast cells resulted in an increased spontaneous apoptosis or rendered the cells highly susceptible to various death-inducing stimuli. Consistent with these results was the finding that neuroblastoma cells, when transfected with a fragment of the human TGase cDNA in an antisense orientation, exhibited reduced susceptibility to RA-induced apoptosis. These observations further support the intent that TGase expression is an essential component of apoptosis. Several studies have revealed that retinoids are general and potent physiological inducers of TGase. Molecular mechanisms involved in regulation of TGase expression have been extensively studied. By introducing full-length RXR cDNA into RA-resistant HL-60R cells, we have observed that specific expression and activation of RXRs results in a marked increase in TGase expression. RXR-induced increase in TGase expression in these cells is associated with their increased propensity to undergo spontaneous apoptosis.

Isolation and characterization of guinea pig, human, and mouse TGase gene promoters provide the important tools for elucidating regulatory mechanisms of TGase expression. Analysis of 5' upstream nucleotide sequences in TGase gene reveals that a functional TGase promoter contains potential responsive elements and binding sites for multiple factors, including glucocorticoid, IL-6, AP-1 and AP-2. Functional analysis of human TGase promoter reveals that its core region, which includes a TATA box, four SP1 sites, and four potential NF-1 sites within 134 bp upstream of the translation start site, is sufficient for accomplishing high constitutive transcriptional activity. These investigators have further shown that four SP1 sites contribute most significantly to high basal promoter activity. Interestingly, the sequences corresponding to RAREs are not identified in either guinea pig or human TGase promoters, suggesting that RA-dependent upregulation of TGase may be mediated outside this region. However, the results obtained by Nagy et al. reveal that cis-acting elements necessary for directing retinoid-dependent transactivation of mouse TGase are embedded within the proximal 3.8 kb DNA that flanks 5' end of TGase gene. Deletion analysis of the promoter led to identification of two regions critical for retinoid-dependent activation of TGase. The most upstream one, located 1.7 kb upstream of transcription start site, is retinoid response element (mTGRRE1) since it contains a triplicate retinoid receptor binding motif. Coupled with a short DNA segment approximately 1 kb upstream of transcription start site, mTGRRE1 mediates full promoter activation by retinoic acid.

At the molecular level, it has been recently shown that tRA-induced expression of TRAIL, a cell surface ligand for death receptors, is responsible for its apoptotic activity in NB4 cells. Since TRAIL induction is rather a late event occurring only after 48 hr of tRA treatment, it is unlikely that TRAIL is directly induced by tRA. Interestingly, earlier events following tRA treatment include induction of certain anti-apoptotic pathways such as, induction of a BCL2 homologue, BCL2A1 and activation of NFκB. These observations suggest that even though the end point is death, tRA may modulate survival and death of myeloid cells in a temporally complex way.

In conclusion, apoptosis, which is a normal fate of many somatic cells, especially in the hematopoietic system, may be a tightly controlled event by retinoids. Thus, retinoids may regulate the rate at which these cells undergo differentiation and apoptosis, depending on the RARs or RXR subtype expression. A precise understanding of events leading to these processes is likely to give new insight into normal and abnormal leukocyte survival. The experimental evidence discussed in this review suggests that activation of RARs induces the genes linked to cellular differentiation; while activation of RXRs induces genes linked to apoptosis. Further understanding of such pathways that selectively control the cell growth and differentiation functions, may suggest ways to control cell survival in diseases ranging from aplastic anemia to leukemia.

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