Acute promyelocytic leukemia (APL), the M3 subtype of acute myeloid leukemia, is one of the most successful examples of translational research in medicine, since the coordinated combination of laboratory and clinical studies has transformed this leukemia from a fatal into a curable disease. In particular, the introduction of all-trans retinoic acid (ATRA) in APL therapy opened a new page in the history of tumor treatment, since this agonist is capable of inducing morphologic and functional maturation of APL blasts. In both APL blasts and APL-derived cell lines, ATRA-induced progression of promyelocytes to a more mature state is mediated through a complex regulation of gene transcription. The events mediated by proteins codified by ATRA target genes that account for the complex and integrated network of intracellular signaling pathways responsible of the completion of maturation, are still largely unknown. In the last few years, the application of siRNA procedures to the study of molecular mechanisms that lead tumor myeloid precursors to mature along the granulocytic lineage has permitted to establish the specific role of a wide range of intracellular signaling molecules in a wide range of cellular functions, including cell cycle regulation and control of gene expression. In particular, for some of these, as PLC-β2 and Vav1, it was established a role in promoting maturation of APL-derived promyelocytes and in regulating the modifications of the cytoskeleton architecture that accompany maturation related motility and migration. The identification of molecules that play crucial roles in the differentiation of APL cells will, ultimately, allow recognizing new possible targets for future therapies of APL patients.

Key words: Leukemia, promyelocytic, acute - Neutrophil differentiation - RNA, small interfering.

Acute promyelocytic leukemia

Acute Promyelocytic Leukemia (APL), the M3 subtype of acute myeloid leukemia according to the French-American-British (FAB) classification, is characterized by a maturation block of myeloid precursors leading to an accumulation of abnormal heavily granulated promyelocytes in peripheral blood and bone marrow. The genetic hallmark of more than 95% of APL patients is the balanced reciprocal translocation t(15;17)(q22;q21), involving the genes encoding for the retinoic acid receptor-α (RARα) and for the promyelocytic leukemia protein (PML) and resulting in expression of the chimerical protein PML/RARα. RARα, a member of the retinoic acid nuclear receptors family, acts as a ligand-inducible transcription factor by binding to specific response elements (RAREs) at the pro-
moter region of target genes. PML, detected in the nuclear bodies multiprotein complexes, belongs to a family of proteins implicated in tumor suppression and control of genomic stability. In particular, PML controls p53-dependent induction of cell apoptosis, growth suppression and senescence in response to ionizing radiation and oncogenic transformation.

The fusion protein PML/RARα is able to form heterodimers with retinoid X receptors (RXRs) that inhibit the transcription of genes essential for granulocytic differentiation. The molecular complexes directly bind to RAREs in their regulatory regions and then recruit co-repressors that induce chromatin condensation and transcriptional suppression. In addition, PML/RARα is able of recruiting the methylating enzymes Dnmt1 and Dnmt3a, leading to the hypermethylation of promoter sequences on retinoic acid downstream genes, whose transcription is thus repressed.

APL, that constitutes about 10% of AML, is one of the most successful examples of translational research in medicine. In the last decades, an extraordinary combination of laboratory and clinical studies has contributed to transform this disease from a fatal into a curable acute leukemia. Moreover, APL represents a model to understand key mechanisms of leukemogenesis and a paradigm for innovative treatments of myeloid leukemias.

**APL therapy**

The primal APL treatments were based on chemotherapy, aimed to inhibit the proliferation of malignant cells. In the early 1980s, the introduction of all-trans retinoic acid (ATRA) opened a new page in the history of APL since this agonist is capable of inducing morphological and functional maturation of APL blasts. The conformation changes of the fusion protein PML/RARα, induced by pharmacological concentrations of ATRA, are at the basis of this phenomenon. This results in the dissociation of the co-repressor complex from RARα, the ATRA preferred receptor, and in the recruitment of a co-activator complex opening the chromatin structure and relieving transcriptional repression. The resulting transcription activity modulates a large number of genes encoding proteins involved in the completion of granulocytic differentiation, such as granulopoiesis associated transcription factors, cytokines/cytokine receptors, as well as their downstream signal transduction molecules. ATRA also induces a caspase-mediated degradation of PML/RARα by activating ubiquitin/proteasome systems dependent on the binding of SUG-1 in the AF-2 trans-activation domain of RARα.

Despite the use of ATRA constitutes a great advance in the therapy of APL, inducing higher remission rate and overall survival in comparison to chemotherapy alone, the ATRA based therapy requires several administrations that are often responsible of undesired side effects such as the differentiation associated syndrome as well as of resistance to retinoids.

The need of novel therapeutic approaches in the treatment of APL patients has introduced, about 10 years ago, As2O3 (ATO), extensively used in traditional Chinese medicine. Currently, ATO represents an effective agent to induce complete remission in patients with relapsed and refractory APL, as well as in patients with newly diagnosed APL. Furthermore, the use of ATRA/ATO combinations brings much better results than the two drugs used alone, in terms of remission and of disease-free survival status. ATO, at variance with ATRA, does not act through nuclear receptors but, even if with a different mechanism in respect to retinoids, induces the degradation of the leukemogenic protein PML/RARα.

In the last few years, several new agents with potential activity in inducing remission of APL are being studied. Among these, Tamibarotene, a synthetic retinoid recently approved for treatment of relapsed or resistant APL. Compared to ATRA, Tamibarotene is more stable and more potent as inducer of APL blasts differentiation. In about 40% of patients not responsive to this molecule, the inefficiency seems to be due to genetic mutations of RARα, its specific nuclear receptor.

Modern approaches to APL therapy include the combination of ATRA administration and anthracycline-based chemotherapy. In particular, the use of calicheamicin conjugated with a monoclonal antibody directed against CD33 (GO, Gemtuzumab Ozogamicin) takes advantages from the high expression of this surface antigen on APL cells. Engagement of GO by CD33 results in its internalization and hydrolytic release of calicheamicin which, in turn, causes irreversible DNA damages and cell death. Preliminary reports have highlighted the sensitivity of APL to GO administered in combination with other agents, like conventional chemotherapy, ATRA and ATO.
Differentiation treatments of APL-derived cells

The elucidation of the intracellular mechanisms leading to granulocytic differentiation and the identification of molecules whose expression and regulation are altered in APL cells constitute an important issue to identify new therapeutic approaches directed to restore the function of the deficient/modified proteins.

The majority of the studies aimed to clarify the molecular mechanisms involved in the completion of the maturation program of APL blasts were performed on APL-derived cell lines, suitable in vitro models to investigate the intracellular signaling pathways activated by differentiating agents.

HL-60 is an early promyelocytic cell line derived from a patient with APL that, despite lacking the typical chromosomal translocation, responds to micromolar doses of different agonists by differentiating along myeloid lineages. The more reliable in vitro model for the study of APL is constituted by NB4, a cell line derived from a patient with APL, that, at variance with HL-60, shows the t(15;17) translocation. In both cell lines, ATRA treatment induces the progression of maturation along granulocytic lineage; however, only NB4 cells reach a neutrophil-like phenotype, as demonstrated by the high levels of surface antigens expression and by the acquisition of a fully polyploid nucleus.

Also low doses of ATO induce a partial differentiation of both the APL-derived HL-60 and NB4 cells, while high concentrations of this molecule inhibit cell growth mainly through apoptosis. Furthermore, a synergism between ATRA and ATO in inducing granulocytic differentiation has been observed in NB4 cells as well as in NB4-derived cell lines resistant to one of the two agonists.

As in APL blasts, ATRA-induced progression of APL-derived cell to a more mature state is mediated through a well known regulation of gene transcription. Integrated approaches including cDNA microarray, proteomics and computational biology, revealed that ATRA-induced differentiation involves essentially transcriptional remodeling, while the effects of ATO reside mainly at the proteome level, creating a molecular basis for the synergistic effects between ATRA and ATO.

At any rate, the events mediated by proteins codified by ATRA target genes, that account for the complex and integrated network of intracellular signaling pathways responsible of the completion of maturation, are largely unknown.

RNA interference to understand the specific role of signaling molecules

Currently, an effective method to quickly analyze the functions of specific proteins in a wide variety of cellular processes is furnished by RNA interference (RNAi) technology. RNAi is a process in which double-stranded RNA suppresses expression of a target protein by stimulating the specific degradation of its mRNA. In particular, this is a multistep procedure in which small interfering RNAs (siRNAs) of 21–25 nucleotides, chemically or enzymatically produced in vitro, are introduced inside the cells by transfection procedures. siRNAs are then incorporated into RNAi targeting complexes known as RISC (RNA-induced silencing complex) that destroy mRNAs homologous to siRNA. To identify the optimal target mRNA sequence, ended to ensure specificity and to minimize off-target effects of siRNA, several tools are now available. Since, in general, it is not possible to predict the optimal siRNA sequence for a given target protein, a mixture of multiple siRNAs needs to be used.

In the last few years, the application of siRNA procedure to the study of the molecular mechanisms that lead tumoral myeloid precursors to maturate along the granulocytic lineage has permitted to establish the role of a number of intracellular signaling molecules involved in a wide range of cell functions, including cell cycle regulation and control of gene expression.

Cell cycle/apoptosis-related molecules

The silencing of the mRNA for phospholipid scramblase 1 (PLSCR1) constitutes one of the first example of the use of siRNA in the study of proteins involved in maturation of APL-derived cells. ATRA treatment induces, in both NB4 and HL-60 cells, increase of the expression of PLSCR1, originally known for its capacity to promote trans-bilayer movement of membrane phospholipids, playing an important role in cell proliferation, maturation and apoptosis. The expression of PLSCR1 during ATRA-dependent differentiation results to be dependent on agonist induced PKC-δ phosphorylation. On the other hand, the decreased PLSCR1 expression by means of specific siRNAs inhibits ATRA induced granulocytic differentiation. This indicates that, although its precise biologic function(s) remains to be determined, PLSCR1, induced upon activation of PKC-δ, is required for ATRA-triggered differentiation of tumoral promyelocytes.
A further example of the use of siRNA in ATRA induced maturation of APL-derived promyelocytes allowed to establish the relationship between expression of UBE1L (ubiquitin-activating enzyme-E1-like protein) and ISG15 (ubiquitin-like species, interferon-stimulated gene, 15-kDa protein), their physical association and coordinate regulation as well as the induced ISG15 conjugation during granulocytic differentiation. ATRA treatment of APL-derived cells induces an increased expression of UBE1L, that triggers PML/RARα degradation and apoptosis. In particular UBE1L associates with ISG15 and induces its conjugation. It was reported that targeting UBE1L mRNA with specific siRNAs inhibited ATRA-induced ISG15 conjugation and, ultimately, the terminal maturation of NB4 cells.

The serine/threonine protein kinase B (PKB)/Akt acts downstream of the phosphoinositide 3-kinase (PI3K) and functions as an essential mediator in many growth-factor-induced cellular responses including metabolism, proliferation and cell survival. The Akt-mediated phosphorylation of the pro-apoptotic Bcl-2 family member BAD, of caspase 9, and of apoptosis signal-regulated kinase 1 (Ask1) inhibits their pro-apoptotic function. The Akt-activity was found to be up-regulated in ATRA-differentiated HL-60 and NB4 cells and the down-modulation of Akt expression by siRNAs in ATRA-treated HL-60 cells reduces the CD11b expression level, suggesting that PI3K/Akt inhibitors may block the anti-tumor properties of retinoids.

It was demonstrated that HL-60 cells over-expressing the focal adhesion kinase (FAK) present a marked resistance against various apoptotic stimuli and do not respond to ATRA treatment. In particular, in FAK over-expressing cells, the ATRA dependent activation of C/EBPα is impaired and this transcription factor does not associate with retinoblastoma protein (pRb), that results hyper-phosphorylated and then inactive. The introduction of FAK specific siRNAs into FAK-over-expressing HL-60 cells resulted in the recovery of sensitivity to ATRA-induced differentiation.

Several other examples of silencing of proteins involved in cell death during agonist-induced granulocytic differentiation of tumoral myeloid precursors have been reported. Several members of Bcl-2 family, like Bfl-1/A1, have been found to be up-regulated by ATRA and correlated with inhibition of apoptosis. The silencing of Bfl-1/A1, by using specific siRNAs, partially restores the sensitivity of ATRA-treated NB4 cells to doxorubicin (Dox) induced apoptosis. These data suggest that increased expression of Bfl-1/A1 in the HL-60 and NB4 cell lines undergoing differentiation contributes to a loss of sensitivity to chemotherapy-induced apoptosis.

It is known that the ligand CDDO (2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid) of the peroxisome proliferator-activated receptor-gamma (PPARγ), member of the nuclear receptor family that forms heterodimers with RXR, enhances pro-apoptotic effects of ATRA in NB4 cells. The silencing of PPARγ mRNA by specific interfering sequences diminished CDDO-induced maturation in NB4 cells.

Recently, the silencing of the death-associated protein kinase 2 (DAPK2), a Ca2+/calmodulin-regulated serine/threonine kinase involved in apoptosis that reaches high expression levels in ATRA-treated NB4 cells, reduces ATRA-induced granulocytic differentiation of this cell line. This was evidenced by lack of morphological changes and by the decreased expression of neutrophil stage-specific maturation genes, such as CD11b, G-CSF receptor, C/EBPα and lactoferrin, during the maturation process.

Down-modulation of MEK1 (mitogen-activated protein/extracellular signal-regulated kinase 1) phosphorylation inhibits proliferation and induces apoptosis of primary AML blasts. ATO and MEK1 inhibition synergize to induce apoptosis in acute promyelocytic leukemia cells. In addition, analogously to Bfl-1/A1, the down-modulation of MEK1 with specific double-stranded RNA oligonucleotides restored ATO sensitivity in a NB4 cell line resistant to ATO-induced apoptosis.

Furthermore, the functional analysis of the human apoptosis-related protein (PNAS-2) performed by means of siRNA in NB4 cells indicate that this molecule has an anti-apoptotic role and participates in leukemogenesis.

About the regulation of cell cycle, only little information was obtained by the use of siRNA technologies. The silencing of the ubiquitin-conjugating enzyme UBE2D3 that is up-regulated in ATRA-treated NB4 cells and mediates ATRA-induced cyclin D1 degradation, leads to blockage of ATRA-dependent cyclin D1 degradation and cell-cycle arrest.

Gene regulation molecules

The mechanisms by which retinoids regulate initiation of mRNA translation for proteins that mediate their biological effects are not well known and the use
of siRNA represents methods to find new information. In particular, expression of the programmed cell death-4 (PDCD4), a tumor suppressor protein that inhibits protein synthesis by suppression of translation initiation, is markedly up-regulated during ATRA-induced granulocytic differentiation of APL-derived NB4 and HL-60 cell lines, in primary APL blasts and in CD34+ hematopoietic progenitor cells. The knockdown of PDCD4 by RNA interference inhibits ATRA-induced granulocytic differentiation and reduces the expression of key proteins known to be up-regulated by ATRA, including p27Kip1 and DAP5/p97, and induced c-myc and Wilms’ tumor 1, but does not alter the expression of c-jun, p21Waf1/Cip1, and tissue transglutaminase (TG2).

Recent studies report that the treatment of APL-derived NB4 cells with ATRA results in the dissociation of the translational repressor 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1) from the eukaryotic initiation factor eIF4E (eukaryotic initiation factor 4E) and in the subsequent formation of eIF4G-eIF4E complexes. By means of the siRNA-mediated inhibition of 4E-BP1 expression it was found that this molecule enhances ATRA-dependent up-regulation of p21(Waf1/Cip1), a protein that plays a key role in the induction of retinoid-dependent responses, functioning as a translational repressor.

Xeroderma pigmentosum group A-binding protein 2 (XAB2) is involved in pre-mRNA splicing, transcription and in transcription-coupled DNA repair, essential for embryogenesis. The knockdown of XAB2 by siRNAs increases ATRA dependent differentiation of HL-60, suggesting that XAB2 is a component of the RARα co-repressor complex with an inhibitory effect on ATRA-induced differentiation.

By means of siRNA based procedures it was also possible to analyze the specific role of transcription factors up-regulated by ATRA during the differentiation process. It was reported that the reduced expression of PU.1 by means of its specific siRNA blocks ATRA-induced neutrophil differentiation of APL-derived cells.

SiRNA technology was also used to study the role of the PML component of the fusion protein characterizing APL. Specific knockdown of PML3, a member of the PML family of cell-growth and tumor suppressor, that activates Cdk2/cyclin kinase activity, indicates a direct role for this protein in the control of centrosome duplication and genome stability.

Finally, depletion of SUMO-3 expression markedly reduces the number of PML-containing nuclear bodies and their integrity, showing that this protein is essential for PML localization and offering new insight into the patho-biochemistry of APL.

### Inositide-related molecules in differentiation of APL-derived cells

Different groups of investigators, including ours, have demonstrated that agonists-induced granulocytic differentiation of APL-derived cells is accompanied by increased expression and activity of several inositide-related signal transduction enzymes as protein kinase C (PKC), PI3K and phosphoinositide-specific phospholipase C (PLC).

The serine/threonine kinases family of PKC mediates a wide number of intracellular functions in different cell types. In particular, PKCs play a key role in regulating the response of hematopoietic cells to both physiological and pathological inducers of proliferation and differentiation. Furthermore, modifications of specific PKC isotypes activity and subcellular distribution have been described by several authors during ATRA-induced granulocytic differentiation of the APL-derived HL-60 cell line. In addition, inhibition of PKC activity induces several caspases to play a crucial role in the initiation and execution of apoptosis in both primary and derived APL cells. Recently, PKCδ was reported to be activated after ATRA-induced differentiation of both NB4 and HL-60 and its inhibition abrogated ATRA-induced differentiation.

Proteins of the PI3K family phosphorylate at the D-3’-OH position of the myo-inositol ring of phosphoinositides leading to produce 3-phosphoinositides, second messengers whose downstream effects are implicated in many processes including regulation of cell cycle, cell transformation, vesicle transport and reorganization of cytoskeleton. A functional role of PI3K activity in neutrophil-like differentiation of HL-60 cells has been reported. In particular, antisense nucleotides strategies to suppress the expression of PI3K regulatory subunit p85 lead to a strong inhibition of ATRA-mediated differentiation of APL precursors. In addition, the ATRA induced up-regulation of PI3Kγ expression and activity as well as of MAPK are both important for differentiation of NB4 cells, suggesting that additive effects of PI3K and MAPK activities are necessary to promote granulocyte differentiation.

PLCs catalyze the hydrolysis of phosphoinositides to second messenger molecules that mediate a variety
of cellular processes including trans-membrane signaling by numerous cell surface receptors.\textsuperscript{40} Production of lipid-derived second messengers also occurs in the cell nucleus, which possesses an autonomous signaling system responsive to external stimuli.\textsuperscript{41} Accordingly, a number of evidence indicates that expression and activity of diverse PLCs isoforms are specifically modulated in both cell and nuclear compartments of differentiating cells\textsuperscript{41, 42} and play crucial roles in agonist induced differentiation of APL-derived precursors.\textsuperscript{43}

**Role of PLC-\(\beta\)2 in agonists-induced granulocytic maturation of APL-derived cell lines**

Among the 13 different isozymes of the PLC family, PLC-\(\beta\)2, firstly isolated from a HL-60 cDNA library, is expressed predominantly in cells of haematopoietic origin.\textsuperscript{40} Its sequence contains a number of functional regions, including a PH-domain that binds to polyphosphoinositides and to cytoskeleton proteins. By means of its catalytic site, corresponding to the highly conserved X and Y domains, PLC-\(\beta\)2 hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) generating the second messengers inositol 1,4,5-trisphosphate (IP\(_3\)) and diacylglycerol (DAG). The long carboxyl-terminal region is involved in the Go\(\alpha\) mediated activation of the catalytic domains and contains a nuclear localization signal.\textsuperscript{40}

PLC-\(\beta\)2 is highly expressed in neutrophils, according to the role that this protein plays in leukocyte signaling and host defences.\textsuperscript{44} PLC-\(\beta\)2 is activated in murine neutrophils after stimulation with fMLP and its activation leads to the production of bactericidal reactive oxygen intermediates.\textsuperscript{44} On the other hand, a deficiency of PLC-\(\beta\)2 induces an impairment of inflammation-related major functions of neutrophils, resulting in a significant reduction of chemotactrant-induced inositol phosphates accumulation, intracellular Ca\(^{2+}\) levels and superoxide production.\textsuperscript{44}

PLC-\(\beta\)2 as a marker of the overcoming of the tumoral myeloid precursors differentiation blockade

Despite PLC-\(\beta\)2 is greatly expressed in neutrophil of peripheral blood, this enzyme is almost absent in primary blasts purified from the bone marrow of patients bearing APL.\textsuperscript{45} The treatment of APL-derived promyelocytes with micromolar doses of ATRA strongly increases the expression of PLC-\(\beta\)2.\textsuperscript{45} In particular, the levels of PLC-\(\beta\)2 expressed by APL blasts after ATRA treatment strikingly correlate with the responsiveness of the APL patients to retinoid-based therapies,\textsuperscript{45} representing a prognostic factor for this leukemia.

The up-regulation of PLC-\(\beta\)2 also characterizes differentiation of primary CD34\(^+\) hematopoietic progenitors along the granulocytic lineage but not the ATRA treatment of AML-M2 derived blasts,\textsuperscript{45} indicating that this protein is a marker to specifically monitor maturation of normal and malignant myeloid progenitors along the neutrophil lineage.

A low but significant dose dependent increase of PLC-\(\beta\)2 expression was also found when APL-derived cells were treated with ATO at differentiating concentrations and this event was particularly evident in a NB4-derived clone only partially responsive to ATRA treatment.\textsuperscript{46} In addition, differentiating doses of ATRA and ATO cooperate to induce differentiation and PLC-\(\beta\)2 expression, in both ATRA sensitive and partially resistant cells.\textsuperscript{46}

As above reported, PML/RAR\(\alpha\)-bound co-repressors are released from DNA after treatment of APL cells with both ATRA and ATO, leading to the activation of genes repressed by fusion protein.\textsuperscript{1} This suggests that, in APL cells, the reduced expression of PLC-\(\beta\)2, whose gene is located on chromosome 15, involved in the t(15;17) translocation, may be related to the presence of the fusion protein. The increased expression of PLC-\(\beta\)2, induced by both ATRA and ATO,\textsuperscript{46} may be related indeed to the removal of the fusion protein that constitutes a common step of the differentiation pathways activated by the two agonists. Therefore, the increase of PLC-\(\beta\)2 expression may be induced by different molecules able to promote the overcoming of the differentiation block of APL cells. In addition, PLC-\(\beta\)2 may be useful to determine the efficiency of new drugs in releasing the block of gene transcription that characterizes acute promyelocytic leukemia.

To investigate a possible role of PLC-\(\beta\)2 in promoting and/or improving the agonist induced maturation of APL derived cells, this protein was positively and negatively modulated during differentiation treatment with ATRA and ATO of both HL-60 and NB4 cells. No effect on differentiation levels were found,\textsuperscript{46} indicating that, even though PLC-\(\beta\)2 expression closely correlates with the differentiation levels
reached by APL-derived cells induced to complete their differentiation plan, it is not able to activate the maturation machinery driving the precursors to the neutrophil-like stage.

Silencing of PLC-β2 mRNA reduces the migration capability and cell cycle progression of tumoral myeloid precursors.

It is well known that PLCs are key molecules in the regulation of several aspects of cell motility. Downstream to PLC recruitment, cytoskeleton reorganization may occur following changes of the pool of inositol lipids, which are able to regulate actin assembly at several levels by interacting with the lipid binding domains of a variety of cellular proteins. In this context, actin cytoskeleton may play important roles in the profound rearrangements of the cell morphology that occur in differentiation of granulocytes and in functional responses of mature neutrophils.

The use of specific siRNAs to counteract the ATRA-induced increase of PLC-β2 allowed to establish that this PLC isozyme, even if ineffective on phenotypical differentiation, affects the differentiation-related motility of NB4 cells. The use of U-73122, an inhibitor of PLC activity highly specific for PLC-β2, supports a key role of PLC-β2 activity in the acquisition of the migratory capability of differentiating tumoral promyelocytes.

In myeloid cells, as in a number of different cell models, specific PLC isoforms regulate crucial phases of the cell cycle. In particular, the serine phosphorylation of nuclear PLC-β1, occurring at the G0/G1 and late G1 phases, seems to be necessary for the progression of the HL-60 promyelocytic cells through the cell cycle. In the same cell model, the DAG generated by nuclear PLC activity is responsible for activating nuclear PKC during the late G1 phase, which is required for the entry of cells into mitosis.

The analysis of the cell cycle distribution of differentiating NB4 cells revealed that the over-expression of PLC-β2 promotes the accumulation of control cells in the S phase and counteracts the increase of cells in the G0/G1 phases induced by ATRA treatment. Accordingly, cells are unable to proceed from the G0/G1 to S and G2/M phases when PLC-β2 expression is inhibited by means of specific siRNAs and its activity is down-modulated by using the specific U-73122 inhibitor. No effects on the expression and the activity of cyclins and cyclin kinases are induced by modulation of PLC-β2, suggesting that other pathways relocate differentiating promyelocytes along the cell cycle.

In differentiating APL-derived promyelocytes, PLC-β2 and its preferred substrate PIP2 interact with the actin component of cytoskeleton and this association seems to be mediated directly by PIP2 via PLC-β2 phosphoinositide-binding domains. The interaction of PLC-β2 with actin is also mediated by PH domain that allows the enzyme to approach its actin-associated substrates.

A number of studies have demonstrated that PIP2 appears a critical regulator of actin polymerization since it up-regulates the activity of many proteins promoting actin assembly and down-regulates proteins that inhibit actin assembly or promote its disassembly. Through the interaction with a number of cytoskeleton proteins, the phosphoinositides, in addition to their classical roles in signal transduction, play important functions in the regulation of cell and nuclear architecture.

Based on phosphoinositide-related mechanisms, PLC-β2 may modulate the amount of PIP2 associated to actin and, therefore, cytoskeleton organization of differentiating tumoral promyelocytes. The resulting modulation of the phosphoinositide pool associated to cytoskeleton may be at the basis of the role of PLC-β2 in modulating migration and cell cycle progression of differentiating promyelocytes, events that both require profound reorganization of cell architecture.

### Role of Vav1 in agonists-induced maturation of APL promyelocytes

Of the Vav family, Vav1 is expressed exclusively in hematopoietic cells and contains an array of structural motifs that allow it to play a role in several distinct cell functions, like proliferation, maturation, cytoskeleton reorganization, regulation of gene expression and apoptosis. The Vav1 domains include a dbl homology (DH) region, whith a guanine nucleotide exchange factor (GEF) activity, a Pleckstrin-Homology (PH) domain, interacting with phosphoinositides, two Src-Homology 3 (SH3) domains that flank on a Src-Homology 2 (SH2) domain, mediating protein-protein interactions and a Calponin-Homology (CH) domain, that functions as an actin-binding domain. To date, the best known function of Vav proteins is their role as guanine nucleotide exchange factors and activators of the Rho/Rac/CDC42 family of small GTPases and
the distinctive functional feature of Vav1 is that its catalytic activity is modulated by the phosphorylation on tyrosine residue/s. GE F activity of Vav1 seems to be directly dependent on inositol lipids, since the binding of PIP2 to its PH domain results in the inability of Vav1 to undergo optimal tyrosine phosphorylation. The substitution of PIP2 with 3-phosphoinositides, resulting by PI3K activity, allows to Vav1 tyrosine phosphorylation.

In the last few years, it has been suggested that some functions of Vav1 in hematopoietic cells are independent from its GEF activity. In particular, Vav1 seems to be involved in the control of gene expression via interactions with chromatin remodelling and/or transcriptional proteins. It is reported that Vav1 associates with the ku70 component of the DNA-dependent protein kinase complex, with homeobox transcription factors and with the heterogeneous nuclear ribonucleoprotein (hnRNP) C. In addition, inside the nucleus of basophilic leukemia cells it was reported that Vav1 is part of a transcriptionally active complex that binds the NPAT binding site of the IL-2 promoter playing indeed a role as adaptor protein involved in regulation of protein phosphorylation or in the assembly of transcriptional complexes.

Stimulation of chemoattractant receptors in non-adherent neutrophils induces a complex sequence of events: actin reorganization, shape changes, development of polarity and reversible adhesion, all culminating in chemotaxis. The complex signaling mechanisms that regulate neutrophil migration are well studied and Vav1 appears to be a major point of the inhibitory crosstalk between adhesion receptors and cytokine receptors. In particular, the activity of Vav1 as GEF for Rac2 is inhibited in adherent cells, possibly due to the activation by adhesion of one or more tyrosine phosphatase responsible of de-phosphorylating Vav1. On the other hand, experiments performed with Vav1/-/- mice demonstrated that neutrophil motility in vitro and neutrophil mobilization into peripheral blood in vivo induced by FMLP were significantly reduced, as well as the generation of filamemtous actin. Intern regulation of neutrophils is also essential for appropriate adhesion and transmigration into tissues. Using Vav1/Vav3-deficient neutrophils, it has been demonstrated that Vav proteins are required for multiple β2 integrin-dependent functions, including sustained adhesion, spreading, and complement-mediated phagocytosis. Vav1 is also implicated in regulating oxidative burst in neutrophils activated by fMLP. In addition, a recent report suggests the intriguing possibility that Vav1 could coordinate Rac and p67phox activation during NADPH oxidase assembly in reconstituted CHO cells, although Vav1-deficient neutrophils and macrophages show no obvious defects in reactive oxygen intermediates (ROI) production in response to a variety of stimuli. Furthermore, Vav1 is also implicated in ROI production downstream of TLR4 and Fc receptors. Neutrophil adhesion is important for oxidative burst induction by various pro-inflammatory agents associated with bacterial infections. In this context, integrin signaling defects in Vav1 null neutrophils have been identified, also accompanied by the absence of ROI production in these cells in response to various adhesion-dependent stimuli.

A functional role of Vav1 was demonstrated also in tumoral myeloid precursors. Recent data have reported the tyrosine-phosphorylation of Vav1 in HL-60 cells upon cross-linking of the human FcγRIIa1 (CD32), that contains an ITAM motif in its intracellular region. A number of evidence indicates the involvement of Vav1 in signal transduction of cytokines or hematopoietic growth factors. In particular, a tyrosine-phosphorylation of Vav1 was reported in G-CSF-stimulated primary human acute myelogenous leukemic cells.

Developmental roles of Vav1 have been described for immune cells in Vav-deficient mice, in which it has been reported that Vav/- immature B and T cells fail to complete their maturation and activation. Even if an increase of Vav1 expression is observed when normal CD34 hematopoietic progenitors are treated with a cytokine cocktail promoting granulocytic differentiation, indicating that an adequate amount of Vav1 has to be achieved along with neutrophil maturation, no evidence indicates for this protein a role in modulating maturation of non-lymphoid cells.

The silencing of Vav1 mRNA counteracts the ATRA-induced maturation of tumoral myeloid precursors. At variance with neutrophils from peripheral blood, that are characterized by a great expression of Vav1, tumoral promyelocytic cells blocked at different steps of their neutrophil differentiation process show variable levels of this protein. The differentiating treatment with ATRA induces a significant increase of the expression of Vav1 in the APL-derived cell lines HL-60 and NB4 as well as in primary blasts obtained from bone marrow of APL patients.
In APL-derived promyelocytes, at variance with normal myeloid precursors, an adequate amount of Vav1 is needed to completion of the maturation process. In fact, when Vav1 is down-modulated during the differentiation treatment with ATRA by means of specific siRNAs, to counteract the increase of the protein induced by this agonist, a significant decrease of the maturation level is reached by treated cells.63

Macroarray analysis performed on differentiating HL-60 cells allowed to assess that down-modulation of Vav1 by silencing its specific mRNA inhibits the expression of 8 genes encoding for cytokine and/or growth factors, induced by ATRA during the maturation process.63 The same results were obtained when tyrosine phosphorylation of Vav1 was specifically inhibited,63 indicating that tyrosine-phosphorylation of this protein plays a crucial role in regulating ATRA-induced maturation of tumoral myeloid precursors along the granulocytic lineage.

Vav1 promotes neutrophil maturation of APL-derived cells

Experiments in which the amount of Vav1 was upregulated allowed to establish that this protein may be directly involved in the completion of maturation program of APL-derived promyelocytes. It was reported that the over-expression of Vav1 in the early promyelocytes HL-60 induces a significant increase of CD11b expression.65 An additional increase of granulocytic differentiation level was found when Vav1 was overexpressed in both HL-60 and NB4 cells induced to differentiate with ATRA.63 Nevertheless, up-regulation of Vav1 is not sufficient per se to induce the achievement of a more differentiated nuclear morphology, which also requires tyrosine phosphorylation of this protein.63

In the APL-derived HL-60 cell line, a number of protein complexes including Vav1 was identified after ATRA treatment, that seems to be responsible of recruiting and activating this protein during the maturation process.64 In particular, a Cbl/Vav1 association was found in the cytoplasm and a SLP-76/Vav1 containing complex was observed in the nuclear compartment of differentiated cells,64 indicating the existence of a compartmentalized association between these molecules during granulocytic differentiation of HL-60 cells, possibly related to a different role of Vav1 in the different cell compartments.

Vav1 may regulate actin cytoskeleton/nucleoskeleton during ATRA treatment of APL-derived cells

In both APL-derived NB4 and HL-60 cells, as in other cell types,55 Vav1 also shows an intranuclear localization and a large increase of tyrosine-phosphorylated Vav1 occurs in nuclei of differentiated cells.63 Syk, a tyrosine kinase interacting with the SH2 domain of Vav1, seems to be responsible for phosphorylate at least one of Vav1 tyrosine residues in differentiating APL cells.64 A pharmacological reduction of the Syk-dependent tyrosine phosphorylation of Vav1 impairs the modifications of nuclear morphology that occur during granulocytic differentiation, indicating that tyrosine-phosphorylated Vav1 plays a crucial role in regulating the nucleoskeleton reorganization of ATRA treated APL derived promyelocytes.64

The best known function of tyrosine phosphorylated Vav1, GEF activity, which causes GTPases-related cytoskeletal changes, seems not to be involved in the ATRA-induced morphological changes in HL-60,65 indicating that alternative mechanisms need to be activated by Vav1 to promote cytoskeleton changes of differentiating promyelocytes.

In ATRA treated HL-60 cells, tyrosine-phosphorylated Vav1 associates with the SH2 domains of the p85 regulatory subunit of PI3K, regulating, in both whole cell and nucleus, the activity of this protein and its interaction with actin.66 When Vav1/PI3K interaction and the related PI3K activity are impaired, by inhibiting Syk-dependent tyrosine phosphorylation of Vav1, the phenotypical differentiation of ATRA-treated HL-60 cells is compromised,65 confirming the notion that PI3K activity is crucial for granulocytic differentiation. In view of the fact that, when the Vav1-dependent PI3K/actin interaction is abrogated, the recovery of 3-phosphoinositides is strongly reduced,65 it is conceivable that the interaction of PI3K with actin may permit the use of its cytoskeleton-associated substrates. This results in modifications of the phosphoinositide pool, that as above reported, play a crucial role in modulating cytoskeleton reorganization.

Concluding remarks

Retinoids and their nuclear receptors activate different signal transduction pathways that cooperate to promote the completion of maturation along the neutrophil lineage of tumor promyelocytes. In APL-derived
cells, a number of ATRA modulated signaling molecules appears involved in the acquisition of a phe-
notypically and functionally mature profile.
The use of small interfering RNAs, that selectively suppress the expression of specific proteins, represents a new valuable tool in the study of APL, since it allows establishing the role of the components of signaling mechanisms activated by ATRA target genes. In addi-
tion, it will help in the identification of molecules useful for the development of new therapeutics approaches to APL.

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