PML/RARA as the master driver of acute promyelocytic leukemia pathogenesis and basis for therapy response

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A B S T R A C T

Acute promyelocytic leukemia (APL) is caused by a chromosomic translocation that always implicates the retinoic acid receptor alpha (RARA) gene. The PML/RARA fusion is by far the most frequent, present in 98% of patients. Over the past 20 years, multiple studies have outlined how PML/RARA interferes with transcriptional regulation and also with assembly of PML nuclear bodies, domains implicated in control of senescence and stem cell self-renewal. However, the respective contribution of each of those defects to APL pathogenesis remains poorly characterized. APL is the model disease for targeted cure of leukemia. Indeed, soon after the demonstration of their clinical activity, retinoic acid (RA) and arsenic trioxide were found to directly target PML/RARA, RA through its RARA moiety, arsenic through the PML one. Analysis of murine APL models has given us an unprecedented level of understanding of the basis for therapy response, highlighting the key role of PML/RARA degradation in the loss of APL self-renewal. Consequently, therapeutic strategies combining RA and arsenic have shown an extraordinary potency in mice and were successfully transposed to patients. While the molecular basis for loss of APL self-renewal remains under study, cure of most patients without any chemotherapy is now clinically achievable.

Learning goals

At the conclusion of this activity, participants should know that:
- PML/RARA is the single APL driver;
- arsenic cures 70% of patients, and its front-line association with retinoic acid cures almost all of them;
- PML/RARA degradation is closely associated with loss of self-renewal and definitive cures.

PML/RARA: the sole APL driver

APL was identified as a separate clinical entity over 50 years ago.[1] One of the key steps in unraveling the disease genetics was the identification of the t(15,17) translocation present in most patients.[2] The latter was characterized at the molecular level in 1990, either through chromosome walking[3] or by direct exploration of the structure of the *RARA* gene,[4] based on the observation of the disease sensitivity to retinoic acid (RA), the ligand of RARA.[5] More than 98% of APLs are associated with the fusion of the promyelocytic gene (*PML*) with *RARA*[6-8] resulting from the t(15,17) translocation (Figure 1). Others are APL patients who harbor alternative translocations involving *RARA*, the most common being t(11;17) that involves the promyelocytic leukemia zinc finger (*PLZF*) gene.[9,10] The constant implication of RARA in these translocations points to a central role of the deregulation of RARA (and nuclear receptor) signaling in APL pathogenesis.

Cancers arise from the accumulation of multiple genetic and epigenetic lesions co-operating to enforce cellular transformation.[11] Leukemias or sarcomas associated with (or defined by) specific translocations may constitute an exception to this model. Indeed, in APL, only rare lesions, often shared with other leukemias or malignancies, have been implicated in progression, such as *MYC* amplification, Fms-like tyrosine kinase 3 activation, or *RAS* mutations,[12,13] findings recently confirmed by pan-genomic approaches in patients or APL mice.[14,15] These do not radically change the presentation of the disease, although activating FLT3 mutations are more often observed in the APLs with hyper-leukocytosis and are associated with a less favorable outcome.[16] The possibility of obtaining transplantable mouse models faithfully recapitulating the human disease, by the mere expression of the PML/RARA transgene in myeloid precursors, provides additional evidence that the fusion protein is the master driver of APL leukemogenesis.[17,18] Human APL has an almost constant incidence with age, suggesting that it arises from a single rate-limiting genetic event.[19] Similarly, studies in APL that develop following chemotherapy have all demonstrated a short (less than a year) time interval between DNA-damaging chemotherapy and disease onset.[20] APL can thus be considered as a quasi-monogenic, X/RARA-driven, disease.[21]

RARA and PML: the constant and major partners of the fusions

Retinoic acid is involved in a variety of physiological regulatory mechanisms, in particular morphogenesis and stem cell self-renewal or myeloid differentiation.[22,23] RARA is a nuclear receptor for RA that exhibits a highly conserved zinc finger-containing, sequence-specific, DNA-binding domain and a complex ligand-binding domain that enable heterodimerization and transcriptional activation.[24] Two other RA receptors have been characterized: RARB and RARG. But surprisingly these have never been implicated in leukemia-associated oncogenic fusions, although RARB was implicated in development of an HBV-driven hepatocellular carcinoma.[25]

RARA is bound to a member of the RXR family of nuclear receptors as an obligatory heterodimer (Figure 1). The RAR and RXR DNA-binding domains each recognize an AGGTCA core motif, in a direct repeat orientation and separated by a spacing of 2 or 5 nucleotides.[26] RARs are versatile transcriptional switches that can either repress or activate transcription. RAR/RXR complexes bind co-repressors in their unliganded state and recruit co-activators in the presence of ligands. Interestingly, RARA appears to be a stronger binder for co-repressors than other RARs.[27]

PML protein initiates the formation of nuclear bodies (NBs), sub-nuclear spherical structures involved in the fine-tuning of several biological processes, such as senescence or stem cell self-renewal, at least in part through the control of P53 signaling.[28] A specific posttranslational modification of PML, sumoylation, controls the recruitment onto NBs of a wide variety of proteins. NBs then modulate the posttranslational modification of these PML partners, resulting in their sequestration or activation[28] (Figure 1). Apart from senescence and stem cell self-renewal, these partner proteins have been implicated in a number of biological and biochemical processes, including DNA repair, apoptosis, or more recently, lipid metabolism (Figure 2).[29,30] Importantly, PML loss is associated with changes in the self-renewal of tissue stem cells, reduced apoptosis and senescence, as well as changes in metabolism.[28,29,31-33]

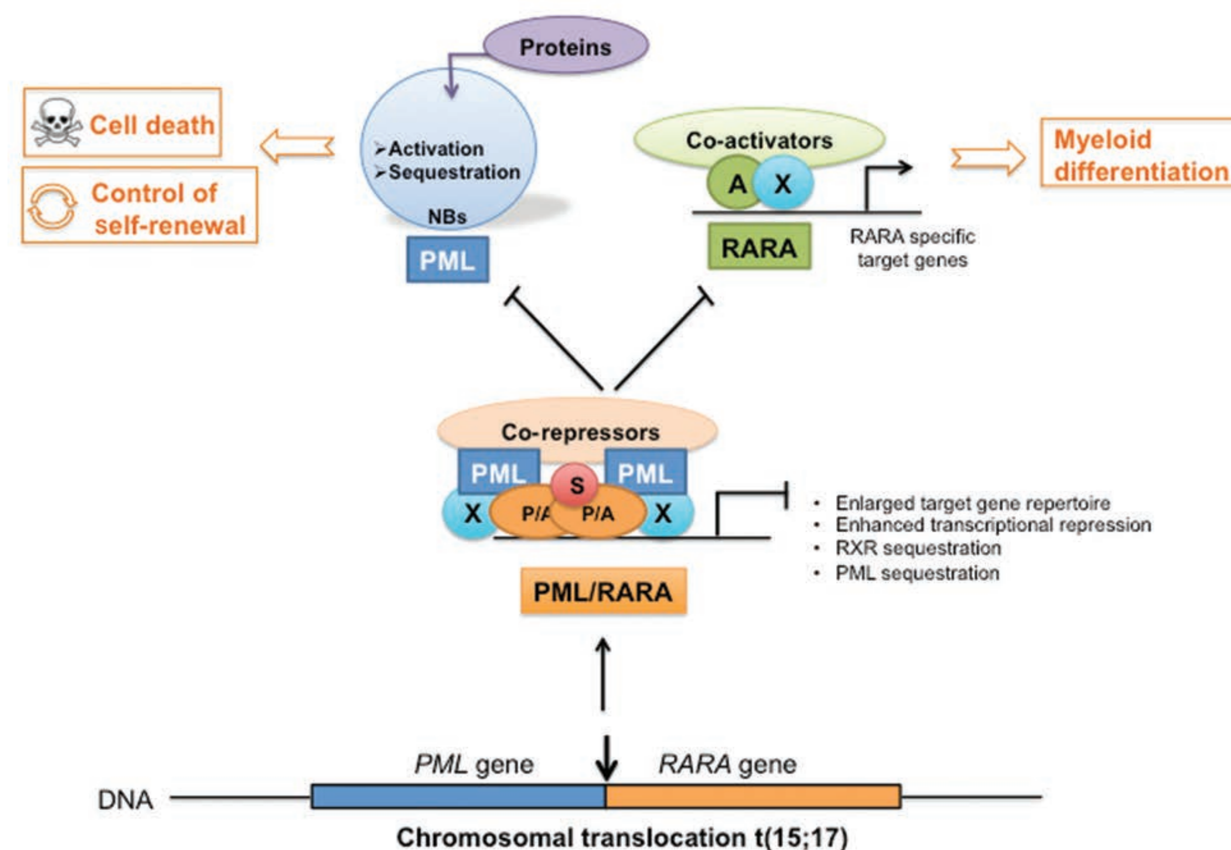

Figure 1. The PML/RARA fusion is a transcriptional repressor that also disrupts PML nuclear bodies. PML/RARA (P/A) binds RXR (X), PML and is sumoylated (S). PML/RARA represses target genes through the recruitment of co-repressors. This blocks RARA (A) targets that are implicated in myeloid differentiation. This also blocks the assembly of PML nuclear bodies, domains that recruit a large number of partner proteins to promote their posttranslational modifications, allowing their activation or sequestration. Defective nuclear bodies were associated to defects in apoptosis control or stem cell self-renewal.

PML/RARA: from a dominant negative to a gain of function oncoprotein

PML/RARA behaves as an altered transcription factor repressing its targets[6] (Figure 1). It was proposed that this results from the ability of PML to impose homo-dimerization to RARA, enhancing its binding to co-repressors and hence the repression of its targets. Interestingly, this capacity of the oncoprotein to self-dimerize is shared by all RARA fusions.[34] In the specific case of PLZF, the most studied RARA fusion partner apart from PML, an additional repression domain was identified in the N-terminus and proposed to explain RA-resistance of this specific subtype of APL.[35] Repression was primarily attributed to recruitment of histone deacetylases, a proposal that was supported by some pharmacological evidence.[36,37] Thus, a simple textbook model emerged whereby PML/RARA behaves as a super-repressor explaining the differentiation block. RA treatment could then release both the transcriptional and differentiation blocks, yielding remissions through induction of differentiation.[38]

Yet, other properties were also demonstrated for PML/RARA, including the ability to sequester PML, RXR, or to regulate transcription from novel DNA-binding sites[39,40] (Figure 1). Further studies shifting from cell lines to *in vivo* models, progressively strengthened the hypothesis that these properties were also important, if not essential, to APL pathogenesis. First, PML/RARA dimerizes with PML, leading to the replacement of the normal speckled nuclear distribution of PML by a micro-speckled one.[41,42] This alteration in nuclear architecture could participate in APL pathogenesis, notably by fostering aberrant self-renewal. Second, in APL cells, PML/RARA is constantly bound to RXRA and RXR-binding is required for *in vivo* transformation.[40,43-45] This PML/RARA//RXRA hetero-tetramer recognizes a wide range of DNA binding sites consisting of 2-3 AGGTCA sites, in any orientation and/or spacing, exemplifying a major gain of function of this oncoprotein.[45,46] Importantly, some of the recognized sequences are targets of other nuclear receptors (VDR, TR, PPAR) controlling myeloid differentiation or stem cell self-renewal. Relaxed binding site specificity through dimerization is a common feature in deregulated oncogenic transcription factors, in particular in myeloid leukemias.[47]

Clarification of the respective contribution of all these features to actual oncogenesis is ongoing. Yet, it should be noted that while in cell lines forced RARA dimerization is sufficient to confer strong repressive ability on RARA signaling and some inhibition of differentiation, attempts to induce APL *in vivo* with RARA dimers were largely unsuccessful.[48] These only succeeded when using the PML dimerization domain,[49] suggesting a key contribution to interference with PML function beyond providing a dimerization interface. Finally, some studies found that the PML moiety itself contributes to transcriptional repression by PML/RARA, through its conjugation by SUMO, a posttranscriptional modification that confers repression ability to transcription factors.[43,50,51] Collectively, while it is evident that deregulation of RARA transcriptional control is a key central feature of APL pathogenesis, the molecular details and respective contributions of the multiple mechanisms proposed remain to be clarified.

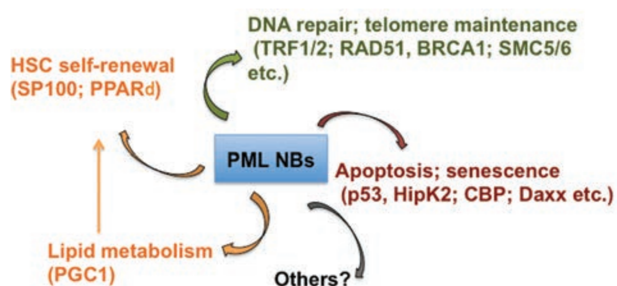

Figure 2. PML nuclear bodies control multiple pathways through modifications of partner proteins. Functions and PML partner proteins associated with them are indicated.

Two drugs for one disease

The introduction of RA for APL treatment in 1985[5] constituted the first example of differentiation therapy.[52] *Ex vivo* and *in vivo*, RA triggers rapid APL cell differentiation into granulocytes, which correlates with patient remissions. With single-agent RA therapy, remissions are usually transient,[53,54] suggesting that differentiation alone cannot abolish cancer cell self-renewal.[21,55] Yet, it should be noted that single agent liposomal RA cured some patients, implying that RA-triggered cure is possible under favorable dosage/pharmacokinetic conditions,[56] in line with mouse models[57] (*see below*).

The other potent anti-APL agent, arsenic, is considerably more efficient than RA as single agent.[58-61] Interestingly, while arsenic is primarily apoptotic *ex vivo*[62] it induces both apoptosis and terminal differentiation *in vivo,* in striking similarity to RA.[21,59] Actually, both agents trigger the so-called differentiation syndrome. As for RA, clinical trials in non-APL cancer patients have been largely disappointing, demonstrating that these compounds exhibit a great specificity for APL cells.[59,63] Such exquisite sensitivity for APL of two completely unrelated agents was puzzling, in particular because arsenic does not control RARA-mediated transcription!

Retinoic acid and arsenic are both PML/RARA-targeted therapies!

Molecular studies performed after demonstration of their clinical efficacy have revealed that both RA and arsenic directly trigger the degradation of the PML/RARA oncoprotein.[21,64-68] In a remarkable and unexpected symmetry, RA targets the RARA part of PML/RARA, while arsenic directly targets its PML part[64] (Figure 3). Thus, these two empirically discovered agents hit PML/RARA through its two constitutive moieties, making them *a posteriori* targeted therapies. This strongly suggested an important, if not essential, contribution of PML/RARA degradation to therapy response.[55,59]

With respect to RA activity, this proposal raised two key issues. What are the molecular mechanisms involved and what are the respective contributions of RA-induced transcriptional activation and degradation to clinical responses. Mechanistically, RA: 1) releases co-repressor binding from PML/RARA; 2) induces AF2-dependent transactivation through the PML/RARA-mediated recruitment of co-activators; 3) induces proteasome-enforced PML/RARA degradation (Figures 3 and 4). In contrast to transcriptional activation, which is already very significant at 10^{-8}M, full degradation requires high RA concentration, presumably because it constitutes a normal feedback mechanism on activation.[67] Accordingly, the therapeutic concentrations of RA required for APL regression are several orders of magnitudes higher than its physiological concentrations, an important observation that was long overlooked. With respect to arsenic, PML/RARA targeting is enforced both by direct binding and by arsenic-induced reactive oxygen species that elicit PML oxidation through the formation of disulfide bridges.[21,70,71] Arsenic targets both PML and PML/RARA. Since these are tightly bound to one another,[39] this dual targeting could enhance response.[70] Therefore, the mechanistic analysis of arsenic activity on APL was intimately linked to the analysis of nuclear body biogenesis. Reformation of NBs and PML degradation occur sequentially.[67,72] As extensively reviewed elsewhere, arsenic-binding and arsenic-triggered oxidation initiate formation of a PML mesh, its hyper-sumoylation, then allowing recruitment of the SUMO-dependent ubiquitin ligase RNF4, which subsequently triggers PML or PML/RARA degradation[72-75] (Figure 3). The role of PML/RARA degradation in arsenic-based therapy is supported by significant genetic evidence. Mutation of the arsenic-binding or arsenic-sensitive sumoylation site in PML/RARA impairs degradation and *ex vivo* response to treatment.[50,70,72] Mutations immediately adjacent to the arsenic-binding site of PML/RARA were observed in arsenic-resistant patients.[76] Finally, vitamin E derivatives with mitochondrial toxicity which, like arsenic, generate oxidative stress, also induce prolonged remissions in murine models of AP.[77] Importantly, arsenic does not induce PLZF/RARA degradation and is accordingly inefficient in PLZF/RARA APL models.[70,78]

Analysis of therapy resistant patients strongly supported these findings. Primary RA-resistance often reflects insufficient levels of RA in the blood, as the result of RA-induced activation of the cytochrome that catabolizes the hormone.[79,80] Then patient cells remain susceptible to RA-induced differentiation *ex vivo*. Some cases of secondary resistance were also linked to mutations in the RA-binding domain in the RARA moiety of PML/RARA.[81,82] They exhibit resistance to RA *ex vivo*. These PML/RARA mutations impede transcriptional activation and degradation, precluding clarification of their respective contributions to therapy response (Figures 2 and 4). Upregulation of cellular export or RA-trapping mechanisms, were proposed to further contribute to decreased RA intra-cellular concentrations. That only pharmacological levels of RA elicit therapy response and full PML/RARA degradation supports an important role for the latter in long-term disease response.[65] With respect to arsenic, mutations adjacent to the arsenic-binding site in the PML moiety of PML/RARA were observed in 2 therapy-resistant patients,[76] although other mechanisms, notably pharmacogenomics, have not been fully explored.[83] Deciphering the respective roles of PML/RARA degradation and transcriptional activation, in an attempt to unify the modes of action of arsenic and RA, was only possible through *in vivo* modeling in mice.

Differentiation and/or self-renewal?

At the cellular level, the concept of differentiation-based therapy in APL primarily relies on the correlation between clinical remissions and morphological maturation of leukemia blasts.[52] However, this cannot explain why only few patients are cured by RA alone, nor why arsenic cures 70% of APL patients, although it does not induce differentiation *ex vivo*. Accordingly, there have been recent controversies as to the exact contribution of cell differentiation to APL cure.[55,84] Studies have addressed this issue by exhaustively examining the effect of therapy, not only on tumor clearance and leukemia cell differentiation, but also on the loss of self-renewal,[55,57] which can only be assessed in transplantation experiments. While it was considered that the first two cellular responses were tightly coupled, recent evidence has dissociated these two end points, and only loss of self-renewal predicts disease eradication *in vivo*.[55,57,84,85] Indeed, in PML/RARA-driven APL, terminal differentiation of the leukemia is achieved even at low RA doses, but complete APL clearance only appears with treatments at the highest concentrations.[57] Similarly, complete loss of clonogenic activity *in vivo* was observed in APL mice treated with the RA/arsenic combination, although the combination actually delays morphological differentiation.[57,78,86,87] Careful examination of PLZF/RARA-driven APLs revealed that they fully differentiate upon RA treatment, while the latter has only modest effects on self-renewal, explaining their clinical RA-resistance and providing the most striking dissociation

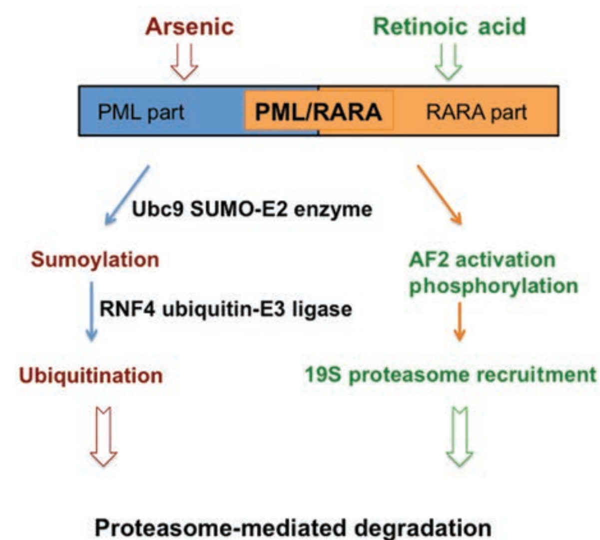

Figure 3. Schematic representation of retinoic acid- and arsenic-triggered PML/RARA catabolism. Note that retinoic acid degrades RARA and arsenic degrades PML.

between APL differentiation and eradication.[57] The fortuitous identification of retinoids that activate RARA-dependent transcription but fail to degrade RARA has provided evidence that only PML/RARA degradation entails loss of self-renewal *ex vivo* or *in vivo,* whereas transcriptional regulation correlates with induction of differentiation[85] (Figure 4). In primary resistance, insufficient RA levels allow differentiation, but not loss of clonogenic activity, resulting in continued APL development. While these observations unify the molecular bases for the antileukemic activity of RA and arsenic (and also explain the potency of their combination, see below), they raise the issue of how arsenic, which does not affect transcriptional regulation, actually induces *in vivo* differentiation. Unpublished evidence from our laboratory has demonstrated that excision of RXRA in APL cells elicits *ex vivo* or *in vivo* differentiation, in the absence of any positive inducer of retinoid signaling. This unexpected result suggests that transcriptional derepression is actually sufficient to trigger differentiation (J Halftermeyer, unpublished observations, 2012). It in turn explains the differentiating effect of arsenic, which clears PML/RARA from promoters, allowing RARA to perform its physiological action.[88] Similarly, the artificial downregulation of PML/RARA (J Ablain, unpublished observations, 2012) or the reversal of histone deacetylation may restore cell maturation processes through mere transcriptional derepression.[89]

What is the basis for loss of clonogenic activity?

PML/RARA degradation entails loss of self-renewal.[85] In principle, full PML/RARA loss should revert all of the proposed effects of the fusion on survival or self-renewal pathways. One of these deserves a particular mention: interference with PML nuclear bodies. Indeed, in normal progenitors or in the context of other leukemic fusion proteins, PML controls self-renewal,[31,32] consistent with the proposal that NBs tune several critical pathways involved in 'stemness' and self-renewal (Figures 2 and 4), such as P53, AKT/PTEN, HIF1A.[90,91]

The triumph of combined approaches

Initial studies performed *ex vivo* demonstrated that RA and arsenic failed to synergize, and even actually antagonize, for APL cell differentiation.[62,86] Yet, as argued above, differentiation is not the most relevant end point to predict clinical efficacy.[55,84,85] Studies performed *in vivo* using genetically defined mouse models or human xenograft, all demonstrated dramatic synergy between these two drugs for survival, through the immediate (3-4 days) loss of self-renewal and clonogenic activity.[57,78,87,92] In retrospect, this can now be

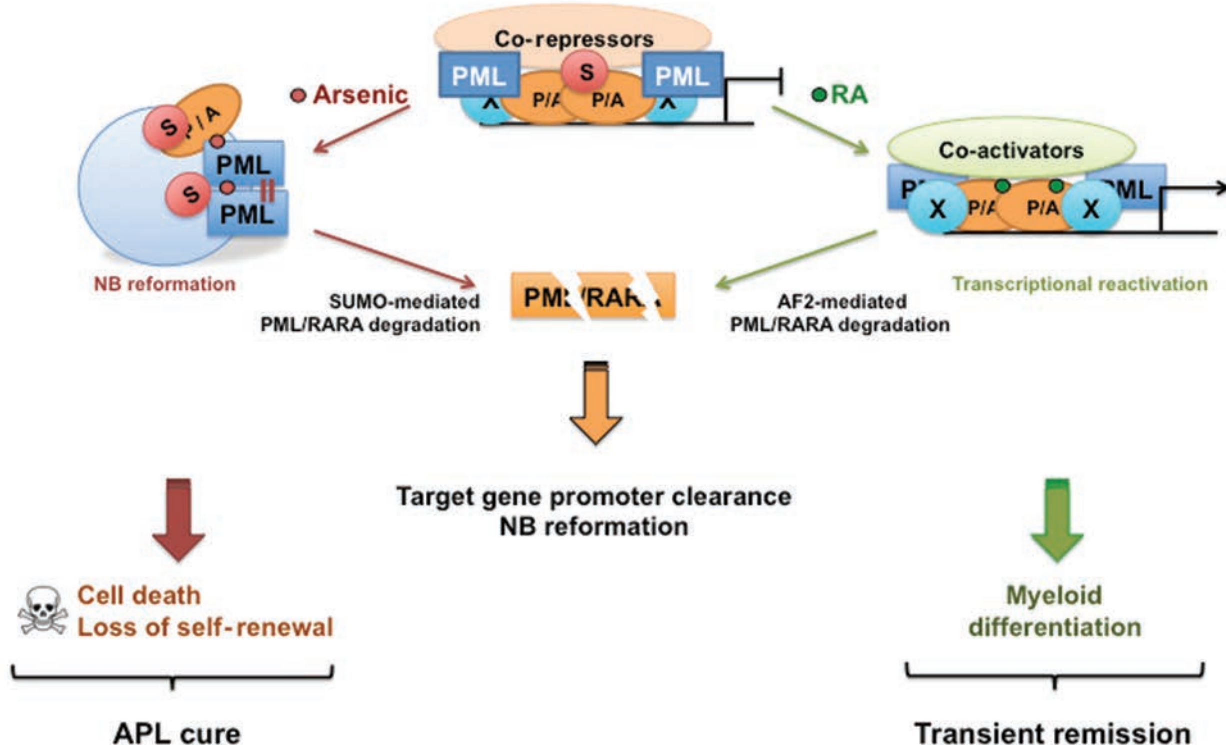

Figure 4. Uncoupling differentiation and cure. (Right) RA activates PML/RARA repressed genes, initiating myeloid differentiation. AF2-mediated degradation also indirectly yields NB reformation. (Left) Arsenic enforces NB reformation, through direct binding and oxidation. NB reformation is tightly linked to loss of self-renewal and apoptosis, correlating with APL eradication. PML/RARA degradation by arsenic also clears promoter and could thus indirectly explain differentiation through promoter clearance. Collectively, through their shared ability to degrade PML/RARA via different mechanisms (Figure 3), both drugs clear target promoters and restore PML nuclear bodies, promoting *in vivo* differentiation and varying degrees of APL clearance.

attributed to the fact that RA and arsenic induce PML/RARA degradation by different mechanisms, predicting accelerated degradation and absence of cross-resistance *in vivo*. In addition, assuming that NB-reformation plays a role in loss of 'stemness', the direct targeting of the normal PML allele by arsenic[70,71] to enforce reformation of NBs may be found to be critical in the eradication process. Front-line combined regimens were successfully transposed to patients, with over 95% of them definitively cured by the association of RA and arsenic[21,54,93-95] (F Lo-Coco, personal communication, 2012), providing a spectacular illustration of the power of mouse models to optimize treatments in patients.[18]

What are the specificities of APL that ensured the success of targeted therapies?

As a paradigm for targeted therapies, APL underscores the superiority of proteolysis over enzymatic inhibition. Indeed, complete degradation abolishes all of the functions of oncoproteins, including those linked to protein/protein interactions, which may be very important in controlling self-renewal.

In APL, the extraordinary clinical potency of RA and arsenic reflects the fact that RARA and PML are both dispensable (in mice), while APL cells are addicted to the continuous expression of PML/RARA. Thus, agents that fully degrade RARA, PML and PML/RARA, exert maximal efficacy on APL cells without any toxicity on normal cells, explaining the high therapeutic index of these agents or their association.[18,21,96] Another reason for the curative activity of these drugs is the great stability of the APL genome, as assessed by next generation sequencing studies.[14,15] Indeed, the APL genome does not seem to be globally instable, contrasting with chronic myeloid leukemias, where resistance to kinase inhibitors gradually occurs as time progresses.[97] Because RA and arsenic degrade PML/RARA by non-overlapping mechanisms, combining RA and arsenic front line reduces the risk of cross-resistance in APL patients. Collectively, the stability of the APL genome, together with rapid tumor debulking by differentiation and the immediate abrogation of all properties of PML/RARA, particularly self-renewal, all contributed to the success of the only example of cancer cure without DNA-damaging therapies.

Diagnosis and monitoring

With the efficiency of the current treatment, the biggest remaining challenge is to reverse the coagulation disorders as early as possible to avoid sudden death through hemorrhage before or in the course of induction. Apart from molecular typing (*see below*), diagnosis may also be achieved through observation of the disruption of PML NBs.[41,98] This highly efficient and straightforward procedure is now used in many centers, as treatment with RA and arsenic can then be started immediately. As in other leukemias driven by fusion genes, PCR on the gene junction has allowed rapid molecular diagnosis, but also the follow up of minimal residual disease. Pioneering studies demonstrated that molecular relapses preceded clinical ones, offering the possibility to re-treat the disease while the leukemic clone remained small. Today, for PML/RARA-driven APLs, the rates of complete remission achieved with current treatments actually question the clinical utility of monitoring the fusion during treatment. This remains an option in the variant APLs for which tools have been recently obtained that have clarified the issue of RA-induced APL clearance in these conditions.[99]

The differentiation syndrome also remains an issue, both with respect to its actual physiopathology and treatment.[100-102] In particular, it is not currently known whether the front-line association of RA and arsenic will decrease its incidence or severity. Intriguingly, how RA reverses the disorders of hemostasis remains to be understood.[103] Finally, the specific issue of hyperleukocytosis at presentation, which still indicates an unfavorable prognosis, should be further evaluated.[104]

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