PLENARY SESSION I
MOLECULAR PATHOGENESIS

RETINOID ACID-INDUCIBLE GENE RIG-I IS AN ESSENTIAL NEGATIVE REGULATOR OF MYELOPOIESIS

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Acute promyelocytic leukemia (APL) is the first leukemia model in which the differentiation arrest of abnormal promyelocytes was clinically rectified by the administration of retinoid acid (RA) at pharmacological doses,[1] then a well-known physiological regulator of vertebrate development.[2] The mystery of this remarkable response of APL cells to RA was betrayed when both PML-RARα and PLZF-RARα oncogenic fusion proteins (hereinafter referred to as X-RARαs), which derives from APL-specific chromosome translocations, were discovered to retain the DNA- and RA-binding domains of retinoid acid receptor α (RARα).[3,4] Subsequently, the oncogenic activity of X-RARαs was then ascribed to their decreased response to RA-induced replacement of transcriptional co-repressors by co-activators onto the promoter regions of RARα target genes.[5,6] However, through repressing what specific downstream genetic programs X-RARαs perturb the normal hematopoiesis has remained largely obscured. About eight years ago, we launched a project to isolate genes whose expression is inducible by RA (retinoic acid inducible genes, *RIGs*) in granulocytic differentiation of APL cell line NB4. It turned out recently, *RIG-I*, a putative RNA helicase, plays an essential role in controlling normal and malignant granulopoiesis. Interestingly, the *Rig-I* expression was found significantly upregulated in RA-induced granulocytic differentiation of both NB4 and AML-M2 subtype HL-60 cells, but not in RA-treated NB4-R2 cells. In line with a notion that the upregulation of *Rig-I* within APL cells may belong to one of events initiated by RA and X-RARα ligation, the *Rig-I* upregulation was apparently hampered in RA-treated hCG-PLZF-RARα bone marrow APL blasts, but realized steadily in those from hCG-PML-RARα transgenic mice. To investigate if *Rig-I* possesses an essential function in modulating normal hematopoiesis, we created a knockout mouse model of *Rig-I* by substituting a neomycine expression cassette for *Rig-I* genomic region spanning Exon4 to Exon 8. At as early as 6 to 8 weeks of age, the percentage of granulocytes in peripheral blood significantly increased, and this abnormality would progress with age. The most unambiguous manifestation of *Rig-I*$^{-/-}$ mice was a moderate splenomegaly, while the cellularities of BM and peripheral blood leukocytes (PBL) were not significantly altered. Nevertheless, the flow cytometric analysis and morphological examination on the individual hematopoietic lineages uncovered an abnormal hematopoietic stem cell (HSC) expansion and a greatly biased granulocytosis ubiquitously evident in BM, spleen and PBL. And an unsaturated Mac-1 expression in *Rig-I*$^{-/-}$ granulocytes, in comparison to the wild type counterparts, together with morphological findings indicates a certain extent of hindered differentiation, which is also evident by the reduced response of *Rig-I*$^{-/-}$ BM granulocytic progenitors to G-CSF-induced granulocytic colony formation. On the other hand, the decreased production of erythroid cells in BM, as shown by the reduced Ter119$^+$ cell compartment size, was compensated by an increased activity of erythropoiesis in spleen, suggesting the occurrence of extramedullary hematopoiesis. The analysis of Annexin V and PI staining of gated BM granulocytes suggested that both reduced apoptosis and increased proliferation contribute to the enhanced granulocytosis in *Rig-I*$^{-/-}$ mice. Of note, the serial transplantation of *Rig-I*$^{+/+}$ and *Rig-I*$^{-/-}$ BM cells into lethally irradiated syngeneic recipients recapitulated this myeloproliferative phenotype, suggesting that this abnormal granulocytosis mostly originates from the autonomous defects in *Rig-I*$^{-/-}$ HSCs. Conversely, the retroviral transduction of *Rig-I* cDNA into 5-FU-treated BM cells of C57BL/6 mice and *in vivo* reconstitution experiment demonstrated that *Rig-I* is a key negative regulator of HSC pool size and granulocytic differentiation. This *Rig-I* deficiency-caused granulocytic predominance in hematopoietic tissues reminded us of the phenotypes caused by PML-RARα and PLZF-RARα transgenic expression[7,8,] whereby an important question arises as to if X-RARαs actually repress the *Rig-I* expression in early myeloid cells, which could contribute to the oncogenic fusion protein-caused myeloproliferative disorder or leukemia. To test this, we enriched early myeloid progenitors from normal and PML-RARα- and PLZF-RARα-transgenic mouse BM samples with magnetic-activated cell sorting, and compared the *Rig-I* expression levels among them. As expected, the mRNA levels of *Rig-I* were significantly reduced in all X-RARα transgenic mice examined. In corroboration to this, X-RARαs, especially PLZF-RARα significantly reduced *Rig-I* mRNA expression in CD34$^+$ compartment of BM cells, which was enriched with malignant APL cells.[9] To further examine the biological importance of this decreased *Rig-I* expression in APL leukemogenesis, conversely, we transduced *Rig-I* cDNA into the freshly isolated APL cells that are passaged *in vivo* via serial transplantation,[10] and infused them into the sublethally irradiated syngeneic recipients. Intriguingly, the survival of the recipients receiving *Rig-I* transduced-APL cells was significantly improved as compared to the control group receiving empty vector transduced-APL cells. All the recipients in control group deceased of APL within one week, before day38 post-transplantation, while half mice in *Rig-I* transduction group survived more than 42 days, and actually 2 surviving mice at end of observation (70 days post-transplantation) were found still health and no sign of granulocytosis in their PBL. In parallel, *Rig-I* transduction significantly induced apoptosis of APL cells over a 48-hour *in vitro* culture. These observations pinpoint *Rig-I* to a potential new target for the treatment of APL as well as perhaps other myeloid leukemias.

As *Rig-I* expression was also reported to be induced by IFN-γ in malignant MCF-7 cells,[11] and the expression of IRF-1, a critical mediator of both RA- and IFN-γ- signaling pathways, has consistently been found to be greatly impaired in APL cases[12,13,] we therefore explored the possibility that *Rig-I* might be a potential target gene of IRF-1, so as the lost activity of IRF-1 in APL may lead to the failure in *Rig-I* transcription. In this regards, we also observed that the reduction in *Rig-I* mRNA level was tightly associated with that of IRF-1 in X-RARα-transgenic mice. In so doing, we inspected *Rig-I* promoter sequence and found a consensus IRF-1 binding site at –113nt of the transcription start site. As expected, the mutation of this putative IRF-1 binding site not only abolished the basal promoter activity of *Rig-I*, but also hampered its activation by IRF-1 overexpression, demonstrating an essentially activating role of IRF-1 on *Rig-I* promoter activity. In summary, our work suggests that *Rig-I* represents the prototype of non-conventional hematopoietic regulators, negatively modulating the normal hematopoiesis and malignant granulopoiesis. Functionally, *Rig-I* appears to be a newly found central integrator of RA-induced and IFN-induced pathways.

Deregulation of *Rig-I* expression or/and its function by X-RARαs is among the first identified critical downstream targets whose *de novo* deficiency causes myeloproliferative phenotype, with enhanced granulocytic growth/survival advantages as well as a moderate differentiation blockage. We believe that our findings yield valuable insights not only into the leukemogenesis of APL and the mechanisms underlying its response to RA, but may also into the pathogenesis of other types of AML and myeloproliferative disorders. The further elucidation of molecular ways surrounding *Rig-I* protein will certainly unveil the new candidates that can be exploited in molecular targeted-therapy of leukemia.

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ANALYSIS OF GLOBAL GENE EXPRESSION IN MODELS OF ACUTE PROMYELOCYTIC LEUKEMIA AND TRANSCRIPTIONAL MODIFICATIONS IN RESPONSE TO RETINOIC ACID THERAPY

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Acute promyelocytic leukemia (APL) is characterized by the 15;17 translocation, which leads to the expression of the PML/RAR fusion protein, an aberrant transcription factor that interferes with the processes of myeloid differentiation.[1] Characteristic of APL is a marked sensitivity to differentiation induced by all-trans retinoic acid (RA) both *in vitro* and *in vivo*.[2] Treatment of APL with RA induces disease remission, and represents the first successful attempt of differentiation therapy that specifically targets the aberrant protein underlying disease onset.[2-4] A peculiarity of APL is the dose-dependent, dual response to RA: PML-RAR expressing cells do not respond to low doses, but have an enhanced sensitivity to therapeutic doses of RA.[5] The interaction of PML-RAR with co-repressor/HDAC complexes is responsible for both transcriptional repression of target genes and sensitivity to RA.[4] PML-RAR functions by de-regulating target genes that are critical to myeloid differentiation, and are thought to represent the downstream effectors of its oncogenic potential.[6] The consequent aberrant transcriptional pattern may result in signaling networks that directly interfere with the normal differentiation program of myeloid precursor cells. RA is thought to act by antagonizing PML-RAR-dependent gene regulation, thereby favoring terminal differentiation. Analysis of the regulatory pathways impaired during leukemogenesis and reactivated during RA-induced differentiation may contribute to the identification of new molecular targets for leukemia therapy. We have used a high-throughput approach to investigate global gene expression profiles in models of acute myeloid leukemogenesis and to study the molecular basis of RA therapy. We first expressed PML-RAR and two other AML-associated fusion proteins (PLZF/RAR, generated by the 11;17 translocation in rare cases of APL and AML1/ETO, generated by the 8;21 translocation characteristic of M2 and M4 AML) in the hematopoietic cell line U937 and measured global gene expression using oligonucleotide chips[7] (Figure 1A).

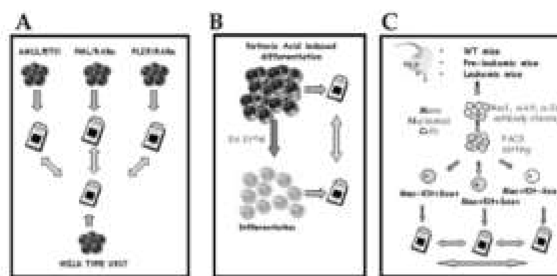

Figure 1.

These experiments showed that a relevant number of regulated genes are targets of more than one AML fusion protein. Functional clustering revealed deregulated activity of genes involved in the control of diverse functions, including differentiation, cell survival, DNA repair, signal transduction and several metabolic pathways. In particular, we found that AML fusion proteins induce genes involved in the maintenance of the stem-cell phenotype and repress DNA repair genes, mainly of the BER pathway. RA treatment causes differentiation of APL cells through PML-RAR-mediated tran-

scriptional regulation. We analyzed global gene expression modifications at an early time point of RA treatment in two models of PML-RAR expressing cells[8] (Figure 1B). First, we assessed gene expression profiles of RA-treated APL blasts with the aim of studying the physiological response to RA therapy, and found significant overlap of results from three different individuals. We then assessed PML-RAR-dependence of transcriptional modulation induced by RA by studying gene expression regulation in the U937 cell system (U937-PR and control U937-Mt cells, prior to and after RA treatment) and found that the vast majority of the identified target genes is modulated by RA only in the presence of PML-RAR. Functional classification of common target genes in APL blasts showed that induction of many specific regulators of differentiation, repression of genes involved in stem cell renewal and/or inhibition of cell differentiation and modulation of genes involved in the regulation of chromatin function are early events in RA-dependent maturation. Some of the repressed genes are involved in multiple hemopoietic stem cell (HSC) functions such as development, maintenance and homing.

We performed a study of the abundance of transcription factor binding sites in the upstream genomic regions and/or the first exons of RA target genes. Surprisingly, putative RAREs are not enriched compared to a random set of genes, whereas recently identified high-affinity PML-RAR binding motifs[9] are strongly enriched in the set promoters of RA target genes. Other specific TFBS display a non-random distribution in the regulatory regions of RA target genes. Some enriched TFBS are bound by transcription factors that are, themselves, encoded by RA target genes. Several TFBS are over or under-represented predominantly in the subset of sequences that contain putative RAREs. This finding suggests that the cis-regulatory capacity of PML-RAR in response to RA may depend on the presence of other TFBS.

The precise nature of the hematopoietic precursor cell targeted by the leukemogenic event is not known, although several investigations indicate that transformation occurs in very early progenitors, most probably in hematopoietic stem cells (HSCs). We have shown that AML fusion proteins induce the expression of stem-cell genes, suggesting a direct link between stem cell molecular networks and leukemogenesis. We are exploiting gene expression profiling to define the molecular signature of selected cellular subpopulations derived from APL mouse models at different stages of disease progression (Figure 1C). In particular, we have analyzed cellular subpopulations selected from the bone marrows of wild type mice or of mice transgenic for PML-RAR in the pre-leukemic and leukemic stages using antibodies directed against three surface antigens (c-Kit and Sca1, specific markers for stem cells, and Mac1, a specific marker for mature myeloid cells). Our results suggest that activation of specific genetic networks that are normally active in HSC is characteristic of PML-RAR-induced transformation, and remain active during disease progression.

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ACUTE PROMYELOCYTIC LEUKEMIA, A MODEL FOR ONCOGENE-TARGETED COMBINATION THERAPY

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APL is rare, but severe condition, since standard chemotherapy rarely yield more than 30% cure rates. In 1988, the group of Pr. Wang showed that this disease could undergo complete remissions upon treatment with retinoic acid through induction of differentiation, identifying the first example of differentiation therapy. In collaboration with L. Degos and A. Dejean, I showed in 1990 that this translocation involves a fusion of the retinoic acid receptor with PML gene to yield a PML-RARα fusion protein. The exquisite sensitivity of the disease to retinoic acid, whose receptor is rearranged, made APL a real paradigm for oncogene-targeted therapies. One should, however, note the highly paradoxical finding of an altered receptor in a disease sensitive to the hormone. It was demonstrated that what was believed to be sensitivity, is in fact relative resistance to retinoic action, since PML-RARα was shown to be a super repressor of nuclear receptor target genes, due to its tighter binding of transcriptional corepressors resulting from PML-induced RARα dimerisation. We have demonstrated that PML is bound to a specific nuclear subdomain and that PML-RARα expression delocalises the components of this domain. These structures, whose exact functions are still unclear are the focus of a great interest having been associated to senescence, transformation, apoptosis or viral infections. Interestingly, $PML^{-/-}$ animal are susceptible to infections, have an impaired apoptosis and are tumour susceptible. Many reports have implicated PML in apoptosis or senescence control, possibly through the control of P53 function. Yet, this is a highly disputed area and the exact mechanism through which PML could control P53 function is unclear. Yet, PML-RARα expression was shown to induce apoptosis resistance and we have identified major and consistent abnormalities of PML expression in a variety of human tumours. Hence, the molecular pathogenesis of APL could be accounted both by a repression of nuclear receptor target genes involved in myeloid differentiation, and by an altered apoptosis control associated with nuclear body disruption. In APL cells, retinoic acid induces both the transcriptional activation of PML-RARα and the relocalisation of PML towards nuclear bodies, linking the repression and NB disruption to the transformed state. Such nuclear body relocalisation results from the degradation of PML-RARα upon RA treatment and likely results in the restoration of the proapoptotic functions of PML. We have analysed the degradation pathways involved in RA-induced PML-RARα catabolism and showed that it involves both a specific cleavage by caspases and the proteasome, through an interaction between the 19S component SUG-1 and the RA-activated AF-2 transcriptional activator function. PML-RARα targeting by RA involves its AF2 domain, both for its transcriptional activation and ligand dependent degradation. Taken the thigh links between activation and catabolism of nuclear receptors and transcription factors at large, it is difficult to separate these two effects. Arsenic trioxide induces a significant number of complete remissions in APL. Arsenic induces both differentiation and apoptosis. The respective contribution of each process to clinical remissions is currently unknown. We have first demonstrated that arsenic, similar to RA, induces PML-RARα degradation and accordingly restores nuclear bodies. In contrast to RA, that targets the RARα moiety of the fusion, arsenic targets its PML moiety, since it also degrades PML. This catabolism is preceded by the targeting of nucleoplasmic PML onto matrix associated nuclear bodies. We have shown that arsenic can enhance the proapoptotic effects of PML, suggesting that the targeting of NB-associated protein such as Sp100, Daxx or p53 onto the nuclear matrix can greatly sensitise the cells to apoptosis. PML can be modified by a family of ubiquitin-related peptides, sumo, in an arsenic enhanced manner. It was proposed that PML sumolation is involved in its nuclear matrix targeting and the recruitment of associated proteins onto nuclear bodies. We have demonstrated that the situation is considerably more complex. Arsenic induces the matrix targeting of PML independently from sumo, sumolation occurs secondarily and is responsible for the recruitement of associated proteins onto the matrix. Among these, we have identified the 11S complex of the proteasome. This is particularly important because PML degradation occurs after sumolation and proteasome recruitment. Hence, sumolation on lysine K160 is a prerequisite for PML degradation induced by arsenic. Our work implies that nuclear bodies could be sites of catabolism, which could account for the astonishing variety of associated proteins. In an attempt to yield an arsenic-resistant APL we have developed transgenic mice where the critical sumolation suite implicated in arsenic response, PML lsyine 160, was mutated. Strikingly, this sumolation site is absolutely required for the APL-specific differentiation block. Viral transduction of hematopoietic progenitors or expression of the fusion protein in trangenic mice fail to recapitulate APL development if this critical sumolation site in PML is mutated. *Ex vivo* studies suggest that the function provided by this sumolation site is transcriptional repression. These observations suggest that PML is the recurrent fusion partner of RARα in APL because, in addition to enforced dimerisation, it fuses a repression domain to the DNA binding domain of RARα. RA-resistant leukemic cells are arsenic sensitive and vice versa. Some groups had described an antagonism between the two agents, at least in cell lines. We have identified a major synergy between the two drugs, both in cell-lines and in an animal model of the disease. In the animal model, combining the two agents suffice to eradicate leukemia. The *in vivo* synergy was later confirmed by two other groups, strongly favouring combined treatments in APL patients. In that respect, data from the Chinese group has demonstrated that blast clearance is greatly accelerated in dually treated patients and that these all reach complete remissions, stressing the relevance of our observations to the cases of APL patients. Moreover, these observations provide the first example where cancer therapy was optimised in the mice and where the optimised treatment was then successfully transferred to patients. More recently, we have shown that cAMP can trigger differentiation of APL cells both *ex vivo* and *in vivo*. Cyclic AMP greatly enhances the differentiation mediated by RA or arsenic. In addition, cAMP can even completely reverse resistance to RA that is conferred by a point mutation in the binding pocket of the RARα moiety of PML-RARα. This likely reflects a cAMP-induced conformational change in the RARα ligand-binding domain and suggests, as demonstrated in embryonal carcinoma cells, that the cAMP and RA signalling pathways both converge onto the same transcription factor: RARα. In fact, a very strong evidence for this model was recently provided by our identification of PML-RARα-specific reporter genes. Using these we have observed that only the combination of RA and cAMP activates/derepresses PML-RARα-dependent transcription in a RA-resistant leukemia, which demonstrates a functional cooperation of these two signalling molecules directly on

PML-RARα. Finally, we have shown that agents that stabilize endogenous cAMP are of clinical value in RA-resistant APL patients. Altogether, our work has shown that the PML-RARα oncogene can be successfully targeted by 3 different agents which directly modulate PML-RARα function and/or stability. Using transgenic mice, that express PML-RARα point mutants known to impair response to these agents, we are analysig the *in vivo* cross-talks between the 3 therapies. The results of this modelling in mice have lead to an optimal treatment that induces durable complete remissions in all patients, providing a decisive model for the future of cancer therapy.

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TWO DECADES OF CANCER GENETICS: FROM SPECIFICITY TO PLEIOTROPIC NETWORKS

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Modeling cancer in mice has reached an even greater relevance in the field of hematological malignancies, due to the already advanced characterization of the molecular basis of many hematological disorders. Important technological developments made it possible to reproduce in the mouse the genetic lesions that characterize human hematological malignancies, thus often generating faithful mouse models of the human condition. These mouse models have also allowed us to develop and test novel therapeutic modalities in pre-clinical studies. However, possibly the most rewarding cultural shift triggered by these modeling efforts stems from what was originally perceived as background noise or modeling inaccuracy. Manipulation of the involved genes often triggered cancer susceptibility in cell types other than the hematopoietic lineages. This prompted us to challenge a fundamental misconception in cancer genetics that the approximately 200 genes directly involved in chromosomal translocations associated with hematopoietic malignancies are specifically and functionally restricted to leukemia/lymphoma pathogenesis only. It has in fact become apparent that these leukemia/lymphoma genes might play a much broader role in cancer pathogenesis than first anticipated, participating in fundamental proto-oncogenic or tumor suppressive networks. Surprisingly, however, there have been very few studies of the role leukemia/lymphoma genes play in other tumor types, despite numerous high-throughput searches for cancer genes involved in solid tumor pathogenesis. We have therefore recently undertaken a pilot systematic study to learn if leukemia/lymphoma genes are indeed implicated in solid tumor pathogenesis. The unexpected and exciting outcome of this analysis will be discussed.