

## REVIEW

# The role of retinoids and retinoic acid receptors in normal hematopoiesis

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**The dramatic therapeutic activity of all-trans retinoic acid (ATRA) in inducing terminal granulocytic differentiation of the malignant promyelocytes that characterize human acute promyelocytic leukemia (APL) has led to numerous studies assessing the role of retinoids and the retinoic acid receptors (RARs) in the regulation of normal hematopoiesis. Studies with knock out mice indicate that retinoic acid receptor activity is not essential for normal hematopoiesis, but both *in vitro* and *in vivo* studies indicate that these receptors may be important modifiers/regulators of different myeloid precursors/progenitors including the primitive transplantable stem cell. A number of target genes have been identified that are either directly or indirectly regulated by RA receptors and which likely play important roles in the retinoid-mediated regulation of myelopoiesis. Several *in vitro* models of hematopoiesis suggest that the transcriptional activity of RA receptors is developmentally regulated during different stages of myelopoiesis. This regulation might involve non-ligand mediated molecular events that alter the interaction of RA receptors with transcriptional corepressor complexes. Moreover, the interaction of RA receptors with other families of transcription factors expressed in different hematopoietic lineages might also account for differential RA receptor activity at different stages of myelopoiesis.**

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## Introduction

The retinoic acid receptors, which include two distinct families, the RARs and RXRs are important regulators of embryonic development and also influence the growth and differentiation of adult cell types. In human acute promyelocytic leukemia (APL) there is a block to normal granulocytic differentiation which, if untreated, results in the lethal accumulation of immature promyelocytes. Virtually all cases of APL are characterized by the presence of a chromosome translocation involving the retinoic acid receptor alpha (RAR $\alpha$ ) gene on chromosome 17.<sup>1–5</sup> The most common of these chromosome translocations, t(15;17) generates the PML-RAR $\alpha$  fusion protein that inhibits the function of normal RARs.<sup>6–8</sup> The block to granulocytic differentiation in APL is overcome by high ‘pharmacological’ concentrations of all-trans retinoic acid (ATRA) which likely accounts for the marked therapeutic effect of ATRA in human APL.<sup>9–12</sup> In addition transduction of a COOH-terminal truncated RAR $\alpha$  harboring dominant negative activity into normal mouse bone marrow generates hematopoietic growth factor dependent cells frozen at distinct stages of myeloid differentiation. These include the GM-CSF-dependent MPRO cells, which consist largely of promyelocytes which can be induced to terminally differentiate to granulocytes with high concentrations of ATRA.<sup>13</sup> Together these observations suggest that the RAR $\alpha$  gene might normally be involved in

regulating granulopoiesis, particularly the terminal differentiation of granulocytes. Here we review pertinent studies that utilize a variety of different *in vitro* and *in vivo* experimental models to determine the role that retinoic acid and the retinoic acid receptors (particularly RAR $\alpha$ ) play in regulating myeloid differentiation

## Retinoic acid receptors and myelopoiesis – *in vitro* models

The dramatic therapeutic activity of ATRA in inducing the terminal granulocytic differentiation of malignant promyelocytes has prompted studies determining what role retinoic acid and retinoic acid receptors might play in regulating normal myelopoiesis. Many of these studies compare the behavior of primitive hematopoietic progenitors cultured in the presence or absence of exogenous ATRA. The target cells for these studies have included normal bone marrow or fetal liver mononuclear cells or more highly purified CD34<sup>+</sup> or lin-c-kit+Sca-1<sup>+</sup> primitive hematopoietic precursors. In these studies the effect of all-trans retinoic acid (ATRA) on the growth and differentiation of these hematopoietic progenitors has included studies both in liquid suspension culture and in semi-solid media (agar or methylcellulose). In general, these studies have suggested a role of ATRA in enhancing the growth and differentiation of granulocyte progenitors.

## *The effect of ATRA on semi-solid cultures of hematopoietic precursors*

A number of different studies have indicated that in semi-solid medium retinoic acid enhances the clonal growth of GM-CSF-dependent colonies derived from normal human bone marrow.<sup>14–18</sup> This enhanced myeloid colony growth was observed with both ATRA and 9-cis retinoic acid.<sup>17</sup> In multiple studies it has been observed that this ATRA-mediated enhancement of granulocyte progenitor growth and differentiation is associated with decreased production of colonies representing other hematopoietic lineages including erythroid (BFU-E),<sup>19–21</sup> macrophage,<sup>18</sup> mixed granulocyte/macrophage<sup>22</sup> and primitive colonies with multipotent potential (HPPs).<sup>21</sup> These latter observations suggest that retinoic acid and retinoic acid receptors might be involved in regulating lineage determination by multipotential hematopoietic progenitors. Consistent with this hypothesis are observations utilizing cytokine-dependent cell lines representing multipotential progenitors that continuously proliferate in liquid suspension. For example, the IL-3-dependent FDCPmixA4 cells harbor mixed lineage potential. GM-CSF induces these cells to undergo granulocytic differentiation, while erythropoietin induces their erythroid differentiation.<sup>23,24</sup> Selective RAR $\alpha$  agonists enhance the myelomonocytic differentiation of the FDCPmixA4 cells, and this is associated with their enhanced RAR $\alpha$  expression.<sup>25</sup> Con-

versely RAR $\alpha$  antagonists, which are synthetic retinoids that bind to RA receptors with high affinity, but do not activate transcription and thus act as competitive inhibitors of RAR activation,<sup>26,27</sup> enhance the erythroid differentiation of these cells while suppressing their myelomonocytic differentiation.<sup>25</sup> Similarly IL-3 enhances the commitment of the multipotential, stem cell factor (SCF)-dependent EML cells to the granulocyte/monocyte lineage,<sup>28</sup> and this commitment is enhanced by ATRA and inhibited by RAR antagonists.<sup>28–30</sup> Thus the observed retinoid-induced enhancement of granulocyte colony formation in cultures of normal bone marrow may reflect a role of activated RA receptors in mediating the lineage commitment of multipotential cells to the granulocyte lineage.

Interestingly, the effect of retinoids on the growth of myeloid progenitors is highly selective for the cytokines utilized in the culture system. In studies utilizing normal mononuclear bone marrow cells or purified CD34<sup>+</sup> hematopoietic precursors, ATRA, when added to clonogenic cultures in semi-solid media, enhanced the generation of IL-3- and GM-CSF-dependent colonies but had no effect or inhibited G-CSF-dependent colonies.<sup>14,31,32</sup> Since G-CSF-dependent colonies likely arise from more mature progenitors than either IL-3- or GM-CSF-dependent colonies, this suggests that ATRA may regulate the proliferation/differentiation of cells at a relatively early stage of myeloid maturation.<sup>33</sup>

#### *The effect of ATRA on liquid suspension cultures of hematopoietic precursors*

The above studies that involve primary hematopoietic cells (rather than cultured cell lines) describe the effect of ATRA on the growth and differentiation of cells cultured in semi-solid media. The effect of exogenous ATRA on the behavior of primitive hematopoietic precursors cultured in liquid suspension in cytokine ‘cocktails’ has also been assessed. These studies also reveal an ATRA-mediated enhancement of GM-CSF-dependent colonies (CFU-GM) in cultures of highly enriched murine hematopoietic precursors (lin<sup>-</sup>c-kit<sup>+</sup>Sca-1<sup>+</sup>).<sup>34</sup> Again ATRA appears to act on a relatively early hematopoietic precursor because enhanced CFU-GM production is not observed in ATRA-treated liquid suspension cultures of more mature precursors (lin<sup>-</sup>c-kit<sup>+</sup>Sca-1<sup>-</sup>).<sup>34</sup> Further evidence that ATRA regulates relatively immature precursors in liquid suspension culture is provided by observations that ATRA enhances the mitogenic activity of CD34<sup>+</sup> cells,<sup>35</sup> generates enhanced numbers of blasts and immature myeloid cells in cultures of lin<sup>-</sup>Sca-1<sup>+</sup> progenitors<sup>36</sup> and enhances the generation of CFU-S and both short- and long-term repopulating cells in cultures of lin<sup>-</sup>c-kit<sup>+</sup>Sca-1<sup>+</sup> precursors,<sup>34</sup> as well as enhancing their serial transplant potential.<sup>37</sup> Moreover, an RAR antagonist when added to liquid suspension cultures of lin<sup>-</sup>c-kit<sup>+</sup>Sca-1<sup>+</sup> precursors markedly inhibits the generation of repopulating stem cells in such cultures.<sup>34</sup> Together these observations suggest that in liquid suspension culture ATRA not only enhances the generation of committed myeloid progenitors but also increases the production of more primitive hematopoietic precursors including CFU-S and both short- and long-term marrow repopulating cells.

The cell/cell interaction in hematopoietic stem cell liquid cultures containing multiple cytokines is likely to be intricate and complex, perhaps involving positive or negative feedback loops influencing stem cell self renewal and/or survival. Indeed it is presently unclear whether the observed effect of

ATRA in enhancing the maintenance and/or production of transplantable hematopoietic stem cells in liquid suspension culture results from a direct effect of ATRA on the stem cells or an indirect effect through ‘accessory’ cells that may regulate stem cell activity in such cultures.<sup>34</sup> Nevertheless, these observations suggest that ATRA might be clinically utilized to expand the number of hematopoietic stem cells in *ex vivo* cultures of primitive hematopoietic precursors and/or to aid in retroviral- or lentiviral-mediated gene transduction of primitive hematopoietic stem cells.<sup>38</sup>

#### **Retinoic acid receptors and myelopoiesis – *in vivo* models**

The above studies assessing the effect of ATRA on cultured hematopoietic cells *in vitro* have generated complex and occasionally contrasting results likely secondary to the large number of experimental variables inherent in such approaches. Such variables include the degree of maturity of the starting target cell population, the cytokines present in the culture media and whether the cultures are performed in liquid suspension or in semi-solid media. Moreover, most of these studies have involved utilizing relatively high ‘pharmacological’ concentrations of retinoic acid (0.1–1  $\mu$ M) which is 100–1000-fold higher than the endogenous ‘physiological’ concentration of retinoids (1–10 nM) normally present in serum.<sup>39,40</sup> Thus the contribution of normal circulating levels of retinoids to hematopoiesis remains unclear. Some of these problems can be overcome utilizing a number of *in vivo* models of retinoid and retinoic acid receptor function including vitamin A deficient (VAD) mice, mice treated with synthetic RAR antagonists,<sup>26,27</sup> and knock-out mice genetically deficient in one or more of the retinoic acid receptors.

#### *Vitamin A-deficient mice*

Retinoic acid is a derivative of vitamin A (retinol) and several models of vitamin A-deficient (VAD) mice have been utilized to determine the role that endogenous retinoids might play in hematopoiesis. Mice of the SENCAR strain when fed a vitamin A-deficient diet develop within 3–5 weeks a severe retinol deficiency that has been previously associated with abnormalities in skin and cervical epithelium.<sup>41,42</sup> Such VAD mice develop an expansion of myeloid cells in the bone marrow, spleen and peripheral blood.<sup>43</sup> These myeloid cells were predominantly granulocytes expressing the Mac-1 and Gr-1 surface antigen profile characteristic of mature, terminally differentiating granulocytes, and no block to their differentiation was noted in these VAD mice. Moreover, *in vitro* colony-forming assays revealed no significant difference in the number of myeloid progenitors between the VAD vs the control mice, and these mice did not show any obvious abnormalities in their numbers of B and T lymphocytes.<sup>43</sup> Importantly, when the diet was again supplemented with ATRA, the myeloid expansion reverted back to normal. The authors suggest that the myeloid expansion in the VAD mice might be related to the reduced spontaneous apoptosis of granulocytes observed in these animals.<sup>43</sup> Indeed retinoids have been previously observed to regulate apoptosis of cultured myeloid cells.<sup>44,45</sup>

A similar myeloid expansion was also observed in cellular retinol-binding protein type I (CRBPI)-deficient mice. CRBPI homozygous knock-out mice harbor low vitamin A stores and develop severe vitamin A deficiency when fed a vitamin A deficient diet.<sup>46</sup> These animals also display an expansion of

neutrophils in the spleen and blood, but in contrast with the observations in the VAD SENCAR mice, the CRBPI<sup>-/-</sup> mice depleted of vitamin A exhibit an increase in the proportion of relatively immature Mac-1<sup>low/+</sup>GR-1<sup>-/low</sup> granulocytes that accompany this myeloid expansion.<sup>47</sup> A similar myeloid expansion associated with an increase in immature granulocytes was also observed in wild-type mice treated with the pan-RAR antagonist BMS493.<sup>47</sup> This expansion of the myeloid compartment with enhanced expression of immature granulocytes observed in the CRBPI<sup>-/-</sup> mice, as well as in mice treated with RAR antagonists indicates that endogenous retinoids play a role in encouraging the terminal differentiation/apoptosis of myeloid cells, an activity that clearly mimics their role in inducing the terminal differentiation of malignant promyelocytes.<sup>9-12</sup>

### RA receptor knock-out mice

In the above vitamin A-deficient mouse models the ligand-mediated activity of all the retinoic acid receptors including the RARs (RAR $\alpha$ ,  $\beta$  and  $\lambda$ ), as well as the RXRs (RXR $\alpha$ ,  $\beta$  and  $\lambda$ ) is presumably inhibited. The effect on hematopoiesis of selectively disrupting individual RA receptors has been explored in knock-out mice. RAR $\beta$  is generally poorly expressed in myeloid cells while both RAR $\alpha$  and RAR $\gamma$  expression is readily detected,<sup>48</sup> and thus the knock-out studies assessing hematopoiesis have concentrated on these latter two RARs. Two isoforms of RAR $\alpha$  (RAR $\alpha$ 1 and RAR $\alpha$ 2) harbor divergent AF-1 domains and are expressed in many different tissues including hematopoietic cells.<sup>49</sup> Mice selectively deficient in the RAR $\alpha$ 1 isoform appear normal with no discernible disorder in hematopoiesis.<sup>48,50</sup> In contrast knockout mice in which both isoforms of RAR $\alpha$  have been disrupted display early post natal lethality and testis degeneration, but do not display any overt abnormality in hematopoiesis.<sup>51</sup> Similarly, RAR $\gamma$  homozygous knock-out mice do not display any abnormality in myelopoiesis.<sup>52</sup> RAR $\alpha$ <sup>-/-</sup>RAR $\gamma$ <sup>-/-</sup> double mutants die *in utero*,<sup>53</sup> and thus examination of hematopoiesis in these animals has been largely confined to the fetal liver. Fetal liver cells from the RAR $\alpha$ <sup>-/-</sup>RAR $\gamma$ <sup>-/-</sup> double mutant animals consist of mostly mature granulocytes that display similar morphology, surface antigen phenotype (Mac-1,GR-1) and gene expression pattern as wild-type mice.<sup>47</sup> Moreover, these animals do not display any enhanced RAR $\beta$  expression that might compensate for the RAR $\alpha$  and RAR $\gamma$  deficiency. Interestingly, both liquid suspension and methylcellulose cultures of bone marrow cells from RAR $\alpha$ <sup>-/-</sup> mice displayed an increase and/or acceleration of granulocytic differentiation suggesting that RAR $\alpha$ , in the absence of ligand might normally be involved in suppressing granulopoiesis.<sup>47,54</sup>

The normal granulopoiesis observed in the fetal livers of the RAR $\alpha$ <sup>-/-</sup>RAR $\gamma$ <sup>-/-</sup> double mutant animals in the absence of RAR $\beta$  expression strongly indicates that the RARs are not essential and are in fact dispensible for granulopoiesis. Thus these studies on knock-out mice together with the studies in the VAD animals clearly indicate that granulopoiesis can occur in the absence of RAR activity, and retinoids and RARs are likely involved in modifying granulopoiesis but are not essential regulators.<sup>47</sup>

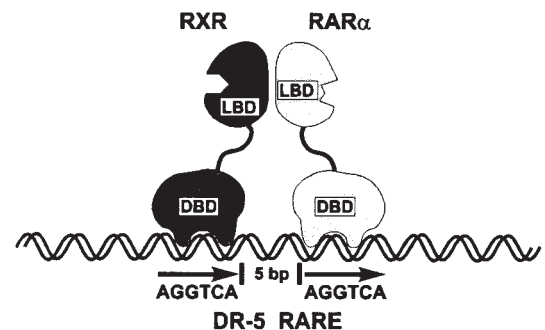
### Target genes for the RA receptors in hematopoietic cells

The retinoic acid receptors are transcription factors which bind as RAR-RXR heterodimers to particular sequences, the

retinoic acid response elements (RAREs) in the regulatory region of specific target genes (Figure 1). These RAREs generally consist of a direct repeat (5' PuGTTCA-3') separated by either 2 (DR2) or 5 (DR5) base pairs.<sup>3,55</sup> What are the target genes for the RA receptors that are likely involved in the RA receptor regulation of both normal and malignant myelopoiesis? A variety of different potential RAR target genes have been identified in different model systems. Indeed studies utilizing expression microarrays have identified extensive changes in gene expression that accompany the retinoic acid-induced differentiation of a number of myeloid cell lines.<sup>56,57</sup> Here we discuss a number of individual genes regulated by retinoic acid that appear to have particular relevance to both normal and malignant myelopoiesis.

### *c-myc*

HL-60 cells were derived from a patient initially diagnosed with acute promyelocytic leukemia<sup>58,59</sup> although a subsequent morphologic assessment indicated that the primary leukemia cells were more appropriately classified as an FAB-M2 leukemia (AML with maturation) rather than FAB-M3.<sup>60</sup> HL-60 cells exhibit a 15–30-fold amplification of the *c-myc* cellular oncogene compared with normal cells.<sup>61</sup> Retinoic acid induces the terminal granulocytic differentiation of these cells,<sup>62</sup> and this differentiation is mediated directly through the retinoic acid receptor.<sup>63</sup> This retinoic acid-induced differentiation of HL-60, as well as other myeloid cell lines, is invariably accompanied by the down-regulation of *c-myc* mRNA expression.<sup>64–66</sup> Moreover, a similar decrease in *c-myc* expression has been observed during the differentiation of normal myeloid progenitors.<sup>67</sup> *C-myc* is a transcription factor involved in regulating proliferation and apoptosis, and enhanced *c-myc* expression is commonly observed in human malignancies.<sup>68,69</sup> Thus the molecular basis for the RAR-mediated down-regulation of *c-myc* expression has been of considerable interest. Nevertheless no RAREs have been identified in the *c-myc* gene promoter suggesting that activated RA receptors do not directly bind to the *c-myc* locus. However, a deletion analysis of the *c-myc* promoter transfected into HL-60 cells induced with retinoic acid identified a retinoic acid responsive element within the P2 promoter of the *c-myc* gene. This element harbors an E2F site<sup>70</sup> which appears to be a critical regulator of *c-myc* transcription.<sup>71,72</sup> Interestingly C/EBP $\alpha$ , another critical regulator of granulopoiesis<sup>73–75</sup> also down-



**Figure 1** The RXR-RAR heterodimers. RA receptors harbor distinct ligand binding (LBD) and DNA binding domains (DBD). The RXR-RAR heterodimer binds to specific gene promoter target sequences, the retinoic acid response elements (RAREs). Here is illustrated a DR5 RARE with a 6 bp direct repeat separated by a 5 bp 'spacer' element.

regulates c-myc expression through this same E2F site.<sup>76</sup> How these different transcription factors might interact with E2F or other molecules to down-regulate c-myc expression at this site is presently unclear.

### *Stra-13*

Stra-13 is a basic helix–loop–helix protein that was originally identified as a gene that was rapidly induced by retinoic acid in P19 embryonal carcinoma cells.<sup>77</sup> Stra-13 expression is associated with growth arrest, as well as diminished c-myc expression,<sup>78</sup> and Stra-13<sup>-/-</sup> mice display lymphoid hyperproliferation with an associated spontaneous activation of T and B cells leading to autoimmune disease.<sup>79</sup> In transient transfection assays Stra-13 down-regulates the c-myc promoter,<sup>78</sup> and thus this retinoic acid-inducible gene may be involved in the down-regulation of c-myc expression that accompanies myeloid differentiation. Although this gene is clearly RA inducible, to date a RARE has not been identified in the Stra-13 promoter (R Taneja, personal communication), and thus whether this gene is directly or indirectly regulated by RA receptors is presently unclear.

### *C/EBPε*

C/EBPε is member of the CCAAT/enhancer binding protein family of transcription factors<sup>80</sup> and is preferentially expressed in terminally differentiating granulocytes.<sup>81–83</sup> C/EBPε<sup>-/-</sup> mice display neutrophil dysfunction leading to opportunistic infections and myelodysplasia,<sup>84</sup> and inactivating mutations of C/EBPε have been observed in individuals with congenital neutrophil-specific granule deficiency.<sup>85,86</sup> Moreover, in certain cell lines G-CSF likely enhances granulocytic differentiation by directly up-regulating C/EBPε expression.<sup>87</sup> Thus C/EBPε appears to be a critical regulator of granulopoiesis. The retinoic acid-induced granulocytic differentiation of promyelocytic leukemia cell lines is associated with enhanced C/EBPε mRNA expression, and a RARE (DR5) has been identified in the C/EBPε promoter.<sup>88</sup> Thus C/EBPε appears to be a direct target of the activated RA receptors. The role played by other hematopoietic transcription factors in regulating C/EBPε expression is presently unclear, and it would be of interest to determine relative expression levels of C/EBPε in the granulocytes of the RARα<sup>-/-</sup> and RARα<sup>-/-</sup>RARγ<sup>-/-</sup> mutant mice.<sup>47</sup> It would also be of interest to determine whether RA receptors can influence granulocytic differentiation in the absence of C/EBPε. Previous studies suggest that RA-induced granulocytic differentiation might ‘bypass’ other important regulators of myelopoiesis. For example although C/EBPα is a critical *in vivo* regulator of granulopoiesis,<sup>73–75</sup> nevertheless cytokine-dependent cell lines derived from C/EBPα<sup>-/-</sup> mice display brisk retinoic acid-induced granulocytic differentiation.<sup>89</sup>

### *Hox family members*

The Hox genes harbor a highly conserved 183 nucleotide DNA-binding homeodomain related to *Drosophila Antennapedia*. The class I Hox genes are arranged in four clusters (A to D) of 9–11 genes/cluster localized on four different chromosomes, and their regulated expression is critical to establishing segment identity during embryogenesis.<sup>90</sup> During hematopoietic development the class I Hox family members, parti-

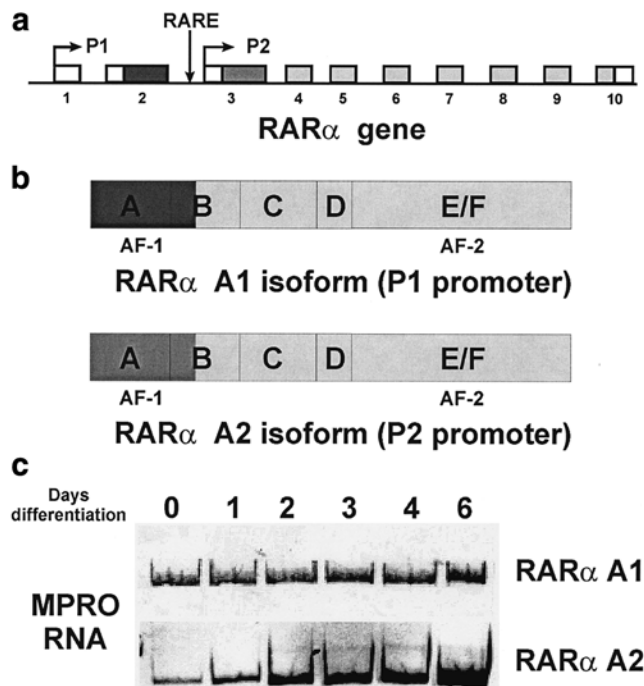
cularly the Hox A and Hox B clusters also display differential expression, with those at the 3′ end generally expressed in relatively immature cells, while those at the 5′ end expressed later in hematopoietic development.<sup>91,92</sup> There is considerable evidence that certain Hox genes are important regulators of hematopoiesis. When overexpressed in hematopoietic cells certain of these genes including HoxB4<sup>93</sup> HoxB7<sup>94</sup> and HoxA10<sup>95</sup> will block differentiation and/or expand progenitor cell numbers both *in vitro* and *in vivo*. Moreover, chromosome translocations involving the HoxA9 gene have been observed in human myelogenous leukemia<sup>96,97</sup> and retroviral insertional activations of both the Hoxa-9 and Hoxa-7 genes have been identified in the BXH-2 mouse model of myeloid leukemia.<sup>98</sup> These observations indicate that the Hox genes might normally play an important role in regulating the proliferation/differentiation of hematopoietic cells. Retinoic acid has been implicated in regulating Hox gene expression during embryogenesis,<sup>99</sup> and in embryonal carcinoma cell lines there is differential sensitivity of the Hox family members to ATRA with those at the 3′ end of the cluster more readily activated than those at the 5′ end of the cluster.<sup>100</sup> Indeed a number of Hox genes harbor well-defined RAREs in their promoters.<sup>101–104</sup> Despite these intriguing potential associations between Hox gene expression, RA receptor activity and the regulation of hematopoietic cell growth and differentiation, there is no clear evidence that the retinoids or the RA receptors are involved in regulating the expression/activity of any Hox family members during hematopoiesis.

### *p21*

The p21<sup>WAF1/CIP1</sup> gene interacts with and inhibits various cyclin-dependent kinase (CDK) complexes that are involved in regulating cell cycle progression.<sup>105</sup> Differentiation is frequently accompanied by exit from the cell cycle, and p21 is frequently up-regulated during the terminal differentiation of a number of different cell types. The retinoic acid-induced granulocytic differentiation of HL-60 cells, as well as the RA-induced monocytic differentiation of U937 cells is associated with enhanced p21 expression.<sup>106–108</sup> The activities of p21 are complex, and at low concentrations this protein enhances active CDK complex formation while at higher concentrations it inhibits CDK activity.<sup>109,110</sup> P21 mRNA expression is enhanced in myeloid colonies derived from CD34<sup>+</sup> cells,<sup>111</sup> and p21 levels progressively rise during cytokine-induced differentiation of CD34<sup>+</sup> cells before falling during terminal differentiation.<sup>112</sup> The p21 promoter harbors an RARE (DR5) suggesting that it is directly regulated by activated RA receptors.<sup>108</sup> Together these observations suggest that p21 may be a critical target gene for RA receptors during granulopoiesis. However, enhanced p21 expression does not appear to be absolutely necessary for regulating terminal granulocytic differentiation since p21<sup>-/-</sup> mice do not have any observed defect in myelopoiesis.<sup>113,114</sup>

### *RARα2 isoform*

There are two isoforms of RARα (RARα1 and RARα2) which completely diverge in their N-terminal AF-1 domain as a result of alternative promoter/exon usage and alternative splicing (Figure 2a, b). Interestingly, the promoter generating the mRNA for the A2 isoform (designated P2) harbors an RARE while the promoter generating the A1 isoform (designated P1)



**Figure 2** Structure and expression of the RAR $\alpha$  gene. (a) The RAR $\alpha$  genomic locus. The P2 promoter harbors a retinoic acid response element (RARE) while the P1 promoter does not. (b) Specific RAR $\alpha$  isoforms (A1 vs A2) harboring distinct AF-1 domains are generated by alternative promoter usage (P1 vs P2). (c) RT-PCR analysis of MPRO cells serially harvested following differentiation induction with ATRA. Primers specific for the RAR $\alpha$  A1 vs the RAR $\alpha$  A2 isoforms were utilized as indicated.

does not.<sup>49,115,116</sup> This promoter structure is evolutionarily conserved as the RAR $\beta$ 2 isoform also harbors an RARE that is not present in the RAR $\beta$ 1 isoform.<sup>117–119</sup> Expression of mRNA for the RAR $\alpha$ 2 isoform is increased during the myeloid differentiation of FDPC mix A4 cells and also accompanies the retinoic acid induced differentiation of NB-4 and HL-60 cells.<sup>25</sup> Moreover, during retinoid-induced differentiation of the GM-CSF-dependent MPRO cells, which provides a robust *in vitro* model for granulocytic differentiation,<sup>13,57</sup> we have observed enhanced expression of the RAR $\alpha$ 2 vs the RAR $\alpha$ 1 isoform (Figure 2c). Together these observations suggest that the RAR $\alpha$ 2 isoform might be a direct target for the activated RA receptor during granulocytic differentiation and that this triggers an autoregulatory positive feedback loop with the RAR $\alpha$ 2 isoform enhancing its own expression. It is possible that the RAR $\alpha$ 2 isoform displays enhanced functional activity compared with the RAR $\alpha$ 1 isoform in differentiating myeloid cells, but this has not been experimentally confirmed.

#### Non-ligand-mediated regulation of RA receptor activity in hematopoietic cells

The above studies indicate that RA receptors are important regulators of myelopoiesis, and that they likely exert their activity by acting as transcription factors to regulate the expression of specific target genes. The RARs were originally identified as members of the nuclear hormone receptor family of transcription factors whose activity was regulated by the addition of exogenous retinoic acid.<sup>120, 121</sup> But if ligand concentration alone is the critical factor in regulating RA receptor

activity, then how is RA receptor transcriptional activity differentially regulated in hematopoietic cells that presumably are exposed to the uniform 'physiologic' concentrations of retinoids that are present in serum (1–10 nM)? One possibility is that different hematopoietic cells metabolize retinoic acid precursors such as retinol (vitamin A) or retinaldehyde at different rates resulting in differences in the intracellular concentration of specific retinoids in different cell types. For example aldehyde dehydrogenase (ALDH), an enzyme that oxidizes retinal to retinoic acid<sup>122</sup> is preferentially expressed in early hematopoietic stem cells.<sup>123,124</sup> However the physiological/biological consequences of this enhanced ALDH expression with respect to stem cell activity is presently unclear. Alternatively, RA receptor activity might be subject to non-ligand-mediated mechanisms of regulation that might be active in cells at different stages of myeloid differentiation. For example, in the relatively immature, multipotent, SCF-dependent EML cells,<sup>28</sup> the transcriptional activity of the RA receptors in response to ligand is relatively blunted, but in the more mature GM-CSF-dependent MPRO promyelocytes<sup>13</sup> there is a marked increase in both ligand-dependent and ligand-independent activity of the RA receptors.<sup>29</sup> These observations suggest that RA receptor activity might be developmentally regulated during different stages of myelopoiesis. The histone deacetylase inhibitor, trichostatin A (TSA), readily activates a retinoic acid responsive reporter in the immature EML cells, but not in the more mature MPRO cells suggesting that there are functionally significant differences in repressor complexes harboring HDAC activity in these different hematopoietic lineages.<sup>29,30</sup> Interestingly TSA also frequently activates reporters driven by RAREs in primary myeloid leukemia samples indicating that repression of RA receptor transcriptional activity may commonly occur in human AML.<sup>125</sup> However, the molecular basis for this inhibition of RA receptor transcriptional activity in primary AML cells is presently unknown.

#### Cytokines and RA receptor activity

Hematopoietic cytokines, particularly IL-3, GM-CSF and IL-1 have been observed to enhance the ATRA-mediated differentiation of primary human APL cells.<sup>126</sup> Moreover, such cytokines appear to be important mediators of RA receptor transcriptional activity, since the addition of IL-3 or GM-CSF to the SCF-dependent EML cells, as well as to normal cultured hematopoietic precursors is associated with enhanced transcriptional activity of the RA receptors.<sup>30</sup> Many cytokines, such as IL-3 and GM-CSF, mediate their biological activities by activating the Jak/Stat pathway. Stats are recruited to activated cytokine receptors where they undergo phosphorylation mediated by their associated JAKs. The activated Stats then translocate to the nucleus where they serve as transcription factors to activate specific target genes.<sup>127,128</sup> Stat family members generally display binding to the consensus sequence 'TTC(N)<sub>2–4</sub>GAA' present in the promoters of their target genes.<sup>129</sup> Recent observations suggest that there may be significant functional cross-talk between the Stat and RA receptor families of transcription factors. For example, the IL-3-mediated enhancement of RA receptor activity observed in EML cells<sup>30</sup> is directly mediated through Stat5.<sup>130</sup> Moreover, there are overlapping Stat/RAR binding sites in the retinoic acid responsive elements (RAREs) of a number of different genes, and Stat5 and RA receptors associate *in vivo* in a cytokine-dependent manner.<sup>130</sup> The nature of the molecular interaction between Stat and RA receptor family members, as well

as their functional consequences are presently unclear. However, such physical and functional interactions between Stats and RA receptors may account for some of the non-ligand-mediated regulation of RA receptor activity that appears to occur at different stages of myelopoiesis.

#### Protein kinase A (PKA) and RA receptor activity

A number of studies have suggested close synergy between PKA and RA receptor activation in triggering the differentiation of myeloid leukemia cells. The retinoic acid-induced differentiation of the HL-60 and U-937 cell lines is markedly potentiated by the addition of agents that increase intracellular cAMP, which is a direct activator of PKA.<sup>131,132</sup> Certain NB-4 subclones that are resistant to ATRA alone exhibit enhanced differentiation when exposed to ATRA plus cAMP analogs.<sup>133</sup> Moreover, certain RXR agonists which are inactive by themselves in inducing NB4 cell differentiation can activate differentiation in combination with the PKA agonist 8CPT-cAMP.<sup>134</sup> PKA phosphorylates RAR $\alpha$  at specific amino acids within the dimerization domain of RAR $\alpha$ , and this appears to enhance RA receptor activity in CV-1 cells.<sup>135</sup> PKA also phosphorylates certain of the RAR transcriptional coactivators and enhances their activity,<sup>136</sup> and this might also explain the synergistic activity of PKA and RAR agonists in triggering the differentiation of certain leukemia cells.

#### Disruption of RAR $\alpha$ activity and the development of promyelocytic leukemia

Human acute promyelocytic leukemia (APL) is associated with different chromosome translocations involving RAR $\alpha$ . At least five different fusion partners for RAR $\alpha$  have been described with the great majority involving either the PML or PLZF genes.<sup>137,138</sup> There is considerable biochemical evidence suggesting that these aberrant fusion proteins function in a dominant negative manner to disrupt the transcriptional activity of the wild-type RAR $\alpha$  gene. In the absence of ligand normal RAR $\alpha$  transcriptional activity is likely inhibited by its interaction with certain corepressors, particularly N-CoR/SMRT, and the addition of ATRA results in the release of such corepressors and recruitment of transcriptional coactivators.<sup>139</sup> The leukemia-specific fusion proteins, including PML-RAR $\alpha$ , display a higher avidity for such corepressors, and significantly higher concentrations of ATRA are required to dissociate the corepressors from PML-RAR $\alpha$  compared with the wild-type RAR $\alpha$ .<sup>6,7,140,141</sup> Interestingly, in contrast with PML-RAR $\alpha$ , the leukemias associated with PLZF-RAR $\alpha$  are generally insensitive to retinoic acid,<sup>142,143</sup> and PLZF-RAR $\alpha$  binding to corepressors is also insensitive to high concentrations of ATRA.<sup>6,7,140,141</sup>

Although the above studies suggest that the RAR $\alpha$  fusion proteins characterizing APL act in a dominant negative manner to disrupt normal RAR $\alpha$  activity, this likely does not entirely account for the leukemogenic activity of these aberrant proteins. Indeed as noted above, the RA receptor knock-out mouse studies indicate that RA receptors are not absolutely required for normal granulopoiesis, raising the question of how blocking RA receptor activity might lead to the block in granulocytic differentiation that characterizes APL. Moreover, while transduction of dominant negative RARs into normal mouse bone marrow leads to the development of cell lines frozen at different stages of myeloid differen-

tiation,<sup>13,28</sup> nevertheless these cell lines are not leukemogenic when injected into syngeneic animals (unpublished). Thus more than just disruption of RAR $\alpha$  activity may be necessary for overt leukemia to occur. Indeed in PML-RAR $\alpha$  leukemias, transformation may result from interference with the activity of both PML and RAR $\alpha$ . PML is a growth suppressor with pro-apoptotic function,<sup>144–146</sup> and this anti-proliferative activity of PML may be related to its functional interaction with p53.<sup>147</sup> The PML-RAR $\alpha$  fusion protein may interfere with this pro-apoptotic activity of PML, and this may be a critical event in the pathogenesis of APL.<sup>148</sup> Similarly in certain myeloid cell lines PLZF enhances growth suppression, and PLZF-RAR $\alpha$ -mediated disruption of this activity combined with its dominant negative effect on normal RAR $\alpha$  may contribute to the leukemic phenotype.<sup>149</sup>

#### Summary

Studies with knock-out mice indicate that the retinoic acid receptors are non-essential and indeed appear to be dispensable for normal hematopoiesis. However both *in vitro* and *in vivo* studies indicate that all-*trans* retinoic acid (ATRA) and the retinoic acid receptors play an important role in modifying/regulating hematopoiesis and may also directly or indirectly enhance the *ex vivo* maintenance/viability of transplantable hematopoietic stem cells. A growing number of target genes have been identified that are directly or indirectly regulated by the activated RA receptors and that are likely directly involved in this regulation of hematopoiesis. Non-ligand-mediated regulation of RA receptor activity appears to characterize different stages of myelopoiesis, and this may involve the interaction of RA receptors with transcriptional corepressors or with other transcription factors such as the Stat family members. This non-ligand-mediated regulation of RA receptor activity may have relevance to the differential sensitivity of human myelogenous leukemia cells to retinoic acid. Paradoxically it is the myeloid leukemia cells that harbor dominant negative retinoic acid receptors (eg PML-RAR $\alpha$ ) that exhibit a therapeutic response to ATRA, while the other types of human myeloid leukemia cells, which generally harbor normal RA receptors,<sup>150</sup> display virtually no response to this agent. Since the malignant phenotype often reflects the underlying phenotype of the normal cells from which the transformed cells arise, defining the molecular basis for the differential activation of RA receptors in normal hematopoietic development may have direct relevance to the question of why some human myeloid leukemias (ie APL) respond dramatically to retinoids while most others (the non-APL leukemias) do not.

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