

## Retinoid Signaling in Cancer and its Promise for Therapy

Krysta Mila Coyle<sup>1</sup>, Mohammad Sultan<sup>1</sup>, Margaret Lois Thomas<sup>1</sup>, Ahmad Vaghar-Kashani<sup>1,2</sup> and Paola Marcato<sup>1\*</sup>

<sup>1</sup>Department of Pathology, Dalhousie University, Halifax, Nova Scotia, Canada

<sup>2</sup>Department of Biology, Uppsala University, Uppsala, Sweden

### Abstract

Deregulated signal transduction is a major facet of cancer development and progression. Herein, we review the current paradigm for retinoic acid signaling, its role in cancer and potential therapeutic applications and challenges. Retinoic acid is used with remarkable success in the treatment of one of the most high-risk leukemias, acute promyelocytic leukemia; however, extending its use in the treatment of other cancers has had limited success at best. Functional studies provide clues for the poor performance of retinoic acid as a general cancer therapeutic, connecting retinoic acid signaling to both cell growth arrest and proliferation with tumor suppression and cancer progression consequences. The dualistic role of the retinoic acid signaling pathway in cancer is revealed in its gene transcription targets, cross-talk with other transcription factors, mediation of apoptotic pathways, and influence in the immune system. If the greatest potential benefit of retinoid-based cancer therapeutics is to be achieved, the many physiological roles of retinoic acid need to be considered.

**Keywords:** Retinoid signaling; Retinoic acid; Therapeutic applications; Chemotherapeutic agent

### Introduction

Retinoic acid (RA) has shown incredible promise as a chemotherapeutic agent in the treatment of acute promyelocytic leukemia (APL). Unfortunately, its use has seen limited success in other cancers, leading to investigations on the role of RA signaling in cancer cells. The promiscuous nature of retinoid-mediated signaling leads to numerous differential downstream effects during RA treatment; thus, we have reviewed literature pertaining to specific contexts of RA signaling and its dysregulation in various cancers. In particular, we note that the effects of RA signaling are largely cellular context-specific. The varied cellular responses to RA treatment may provide an explanation for the failure of RA to reach its full potential in treatment and prevention of solid tumors, including breast, lung, ovarian, prostate, and pancreatic cancers. Indeed, further exploration of the targets of RA transcriptional activity may see improved benefits in clinical studies of cancer treatments.

### Physiological Retinoic Acid Signaling

RA, a metabolite of vitamin A, is a key physiological signal transduction molecule. RA and its related analogues (termed retinoids, Figure 1) play important roles in cell proliferation and differentiation. Retinoids are hydrophobic and lipophilic molecules with cyclic end groups and a conjugated side chain with a polar end group. These properties, coupled with their small size, allow retinoids to enter cells through the cell membrane. Retinoids are thus ideal candidates for medical therapies, as the uptake of RA (and its closely related synthetic analogues) does not require a cell surface receptor [1].

### Vitamin A and its metabolism

Vitamin A, a retinyl ester, is primarily provided by dietary  $\beta$ -carotene, found in colored fruits and vegetables. Approximately 70% of the total retinoids in the body (mostly retinyl esters) are stored in specialized hepatic stellate cells [2]. Retinyl esters are metabolized in the intestine by retinyl ester hydrolases to retinol (Figure 2). Most natural retinoids are transported as retinol, bound to retinol-binding protein 4 (RBP4) [3]. As shown in Figure 2, retinol/RBP4 complexes can undergo receptor-mediated endocytosis via stimulated by retinoic acid gene 6 (STRA6) [4]; or, retinol can be taken up via diffusion [5,6].

Retinol binds cellular retinol-binding proteins (CRBPs). CRBP1 acts as a substrate for intracellular retinol dehydrogenases to oxidize retinol to retinaldehyde (retinal, Figure 2) [7]. After oxidation, the CRBP1-retinal complex is a substrate for RA synthesis by retinaldehyde dehydrogenases (RALDHs), which are also known as aldehyde dehydrogenase 1A1 (ALDH1A1), ALDH1A2, and ALDH1A3 [8]. Cellular RA-binding protein 2 (CRABP2) delivers RA to the nucleus (Figure 2) where it acts as a signal transduction molecule by regulating transcription of many genes [9]. Catabolism of RA occurs via the CYP enzymes [10-12].

### Retinoic acid signal transduction and transcriptional activity

There are multiple physiological isomers of RA with distinct function and binding properties (Figure 1A). The predominant physiological isoform, *all-trans* RA (atRA) activates nuclear hormone receptors (NHRs), retinoic-acid receptors (RAR $\alpha$ , RAR $\beta$  and RAR $\gamma$ ) [13]. The RARs form heterodimers with retinoid-X receptors (RXR $\alpha$ , RXR $\beta$  and RXR $\gamma$ ). RARs can be activated by *all-trans*, 9-cis, and 13-cis RA, however; RXRs preferentially bind and are activated by 9-cis-RA [13].

NHRs recognize hormone response elements (HREs) in DNA; these are namely direct repeats of variations on the hexameric DNA sequence, AGGTCA, separated by one to five spacer nucleotides (DR1-DR5). The heterodimer RAR/RXR binds to specific retinoic-acid response elements (RAREs) in gene promoter regions. The canonical RARE sequence is a direct repeat of the NHR hexameric sequence, RGKTCA, separated by 5 nucleotides. In addition to the canonical

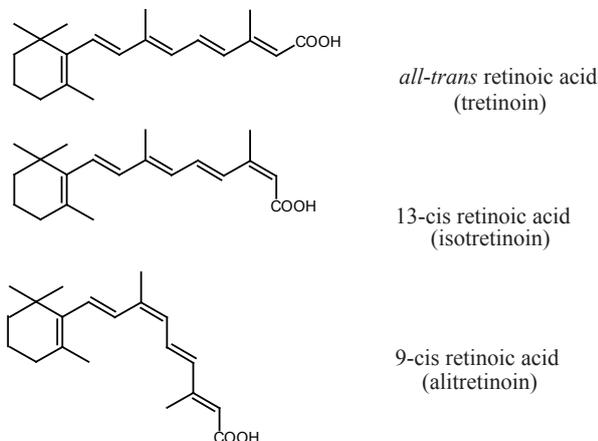
**\*Corresponding author:** Paola Marcato, PhD, Assistant Professor, Department of Pathology, Dalhousie University, Rm 11F11, 5850 College St Halifax, NS, Canada B3H 4R2, Tel: (902) 494-4239; E-mail: [paola.marcato@dal.ca](mailto:paola.marcato@dal.ca)

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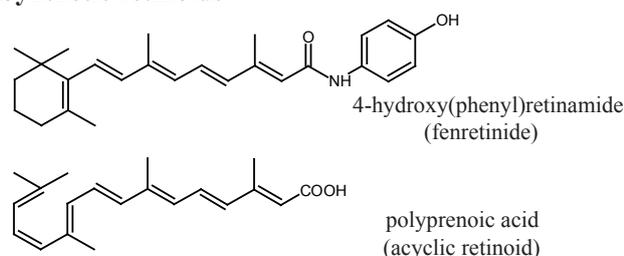
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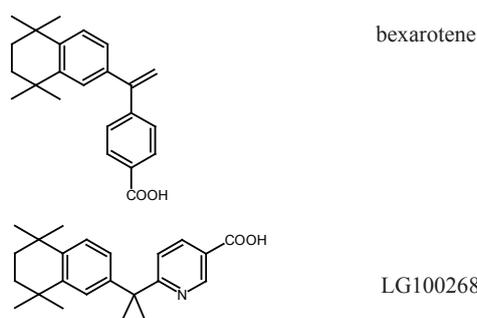
### A Naturally-occurring retinoids



### B Synthetic retinoids



### C Synthetic RXR-specific retinoids



**Figure 1:** Chemical structure of selected physiological and synthetic retinoids. RA exists as several isomers *in vivo* including *all-trans*, 13-*cis* and 9-*cis* RA (A). The physiological isomers as well as the synthetic retinoids (B) have a cyclic end group, a conjugated side chain, and a polar end group, with the exception of polyprenoic acid (acyclic retinoid) which maintains a closed circular shape but is not cyclic. The synthetic retinoids (C) vary in their structure but exist primarily in 9-*cis* conformation. The 9-*cis* configuration (including physiological 9-*cis* RA) supports preferential binding to RXRs.

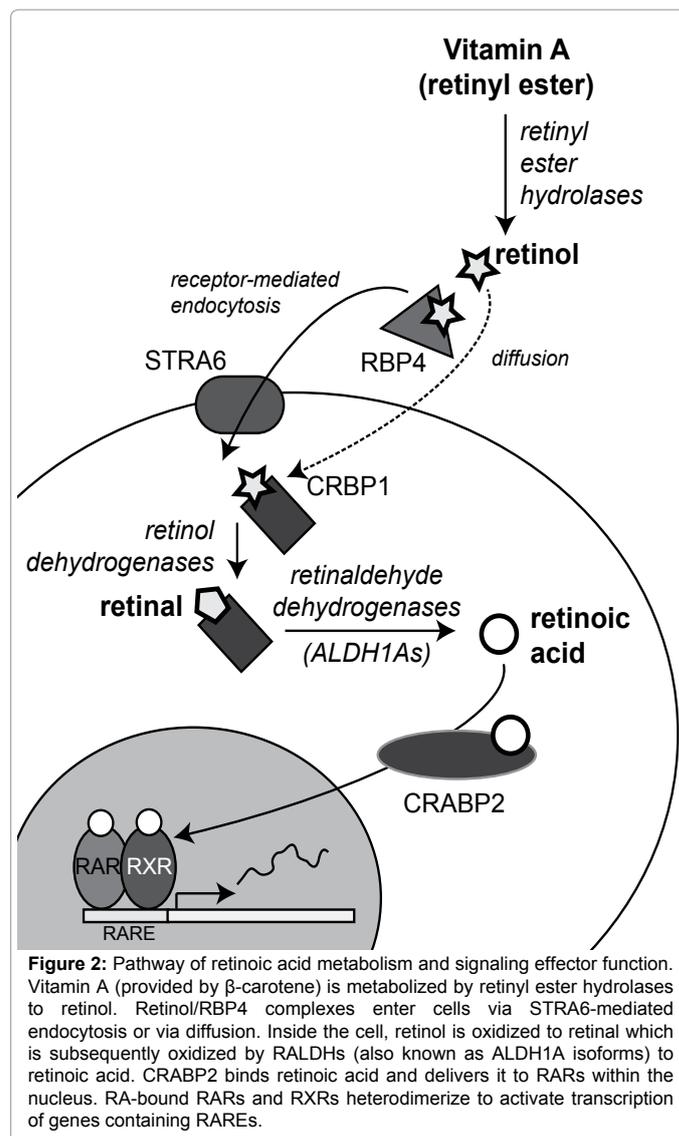
DR5, RAR/RXR have been found to bind to DR0, DR1, and DR2 sites, as well as a composite DR8 [14]. Over 3000 human genes have been found to be associated with RAREs, leading to a wide variety of possible biological functions [15].

Unliganded RAR/RXR heterodimers are typically found bound to DNA with corepressor nuclear corepressor (NCoR) and silencing mediator for retinoid and thyroid receptors (SMRT) [16-18]. NCoR

and SMRT form complexes with histone deacetylases (HDACs) and activate HDAC3 [19-22]. The HDAC activity associated with these corepressors maintains condensed chromatin in the bound promoter, repressing transcription [21,23].

Coactivators bind to NHRs to enhance transcriptional activity upon ligand binding. The RAR/RXR-associated coactivators include the p160 subfamily of steroid receptor coactivators (SRC)-1, SRC-2, and SRC-3 [24], as well as the CBP/p300 coactivator [25-27]. The SRC complex, which contains acetyltransferases and methyltransferases, as well as the histone acetyltransferase activity of SRC-1 and 3 function to open chromatin and allow transcription to occur [28-30].

In addition to ligand-binding-directed transcriptional mediation, a new paradigm for phosphorylation-directed transcriptional mediation, much like other nuclear receptors, has arisen [31]. Evidence which supports this mechanism of action continues to accumulate [32,33]. Phosphorylation of RAR $\alpha$  recruits RAR $\alpha$  to target promoters and enhances recruitment of transcriptional machinery [34]. Additional phosphorylation steps may direct ubiquitin and proteasomal-mediated degradation of RARs [35,36].



RAR/RXR binding to regulatory elements was generally thought to activate transcription; however, a number of studies have identified genes which are downregulated in the presence of retinoic acid [37-40]. Several mechanisms may be possible for this transcriptional repression. Many nuclear receptors share coactivators and corepressors; sequestration or increased availability of these molecules mediated by RARs may affect the activity of other NHRs [41,42]. The potential cancer-related consequences of RAR interactions with other transcription factors is discussed later.

## Cancer-Associated Retinoic Acid Signaling

### Retinoic acid induces tumor suppressors and proto-oncogenes

The wide range of genes which are associated with RAREs suggests that RA can have a large number of biological effects. Therefore, it is unsurprising that RA has a role in cancer which can be promotional or suppressive. RA induces expression of documented proto-oncogenes and tumor suppressors (e.g. mucin 4, MUC4; and retinoic acid receptor beta isoform 2, RAR $\beta$ 2).

RA has been implicated in the expression of several mucins including MUC2, MUC4, MUC5AC and MUC5B [43-45]; aberrant expression of these secreted mucins is also associated with tumorigenesis. A MUC2 knockout model is associated with increased intestinal adenocarcinomas [46], and MUC5B is aberrantly expressed in gastric and breast cancers [47,48]. MUC4, a transmembrane mucin, has also been associated with RA as well as cancer cell growth. RA in combination with transforming growth factor-beta (TGF- $\beta$ ) and interferon-gamma (IFN- $\gamma$ ) can induce the expression of MUC4 by transcription via its RARE [45,49,50], and it is aberrantly expressed in numerous cancers [51-54]. Inhibition of MUC4 reduces pancreatic cell growth *in vitro* as well as tumor growth in a murine model [55,56]. Additionally, MUC4 can induce epithelial-mesenchymal transition (EMT), which is required for metastasis [57]. MUC4 activates human epidermal growth factor receptor 2 (HER2/neu) and promotes signaling by inducing HER2/neu translocation to the cell surface [58-60]. Her2/neu overexpression is typically associated with more aggressive breast cancers via increased proliferation [61,62], angiogenesis by vascular endothelial growth factor (VEGF) induction [63,64], and invasiveness [65,66]. It has also been demonstrated that high levels of HER2/neu confer resistance to atRA in breast cancer cell lines [67]. The cross-talk between MUC4 and HER2/neu is thus an important consideration in using RA as an anticancer drug especially in pancreatic, ovarian, and breast cancers.

RAR $\beta$ 2, one of multiple RAR $\beta$  splice variants, is a well-documented tumor suppressor. RA signaling via RAR $\beta$  promotes apoptosis in human breast cancer cells [68,69]. When RAR $\beta$ 2 is epigenetically silenced, RA is able to promote proliferation of breast cancer cells [70] (Figure 3A). In cancers, RAR $\beta$ 2 is often silenced via histone deacetylation or DNA methylation at its regulatory region (Figure 3A), giving credence to its hypothesized tumor suppressor function [71-74]. Alteration of epigenetic modifications at the RAR $\beta$ 2 locus may be suitable targets for retinoid-resistant cancer therapy [75]. In DLD-1 colon cells, a combination treatment of atRA and 5-aza-2'-deoxycytidine acted synergistically to reduce clonogenicity [76]. In contrast, RAR $\beta$ 2 has also been associated with poor prognosis in non-small cell lung cancer (NSCLC); and, silencing of RAR $\beta$ 2 reduced proliferation and increased apoptosis [77]. This suggests that in addition to its role as a tumor suppressor, RAR $\beta$ 2 may be a tumor promoter in certain scenarios; thus, its role in cancer requires further study.

Retinoic-acid receptor responder protein 1 (RARRES1, formerly

TIG1) is another RA-inducible gene which is gaining notice as a tumor suppressor in many cancer types. Typical of tumor suppressors, RARRES1 is also often silenced in cancers by promoter hypermethylation [78-80]. In nasopharyngeal carcinoma HK1 cells, knockdown of RARRES1 increased proliferation [81]. It has been shown that RARRES1 expression correlated with cellular differentiation in colorectal adenocarcinoma tissues [82]. Additionally, RARRES1 expression is diminished in prostate cancer tissue and forced expression of RARRES1 in PC-3M cells decreased tumorigenicity in mice [83].

It is noteworthy that numerous studies report epigenetic modifications in key components of the RA signaling pathway as well as RA-inducible genes. This suggests that the role of RA in cancer is inseparably linked to epigenetics.

### Retinoic acid signaling in tumor stroma

There is increasing evidence for a significant role of the tumor stroma in cancer progression and metastasis [84-86]. Activation of stromal fibroblasts occurs early in tumor development [87,88], and stromal cells cross-talk with the cancer cells of the tumor, resulting in increased tumorigenesis [89-91]. There is support for aberrant RA signaling via the tumor stroma as well as from cancerous cells. High levels of ALDH1A1 (also known as RALDH1), which generates RA from retinal (Figure 2), is often expressed in the stroma of breast cancer patient tumors and has been correlated with better survival in breast cancer [92]. With respect to expression of RA-inducible genes such as RAR $\beta$  in the tumor stroma, the data is conflicting. Supporting its likely role as a tumor suppressor, RAR $\beta$  expression is frequently lost in the tissue adjacent to tumors [93]. However, confounding this theory, in an ErbB2-induced mammary tumorigenesis model, stromally-expressed RAR $\beta$  is required for mammary tumorigenesis [94]. Not only does this provide evidence of a potential role for RAR $\beta$  in promoting cancer; but, it also illustrates that RA signaling in the stroma can be tumorigenic.

### Interaction with other transcription factors and implications of cancer

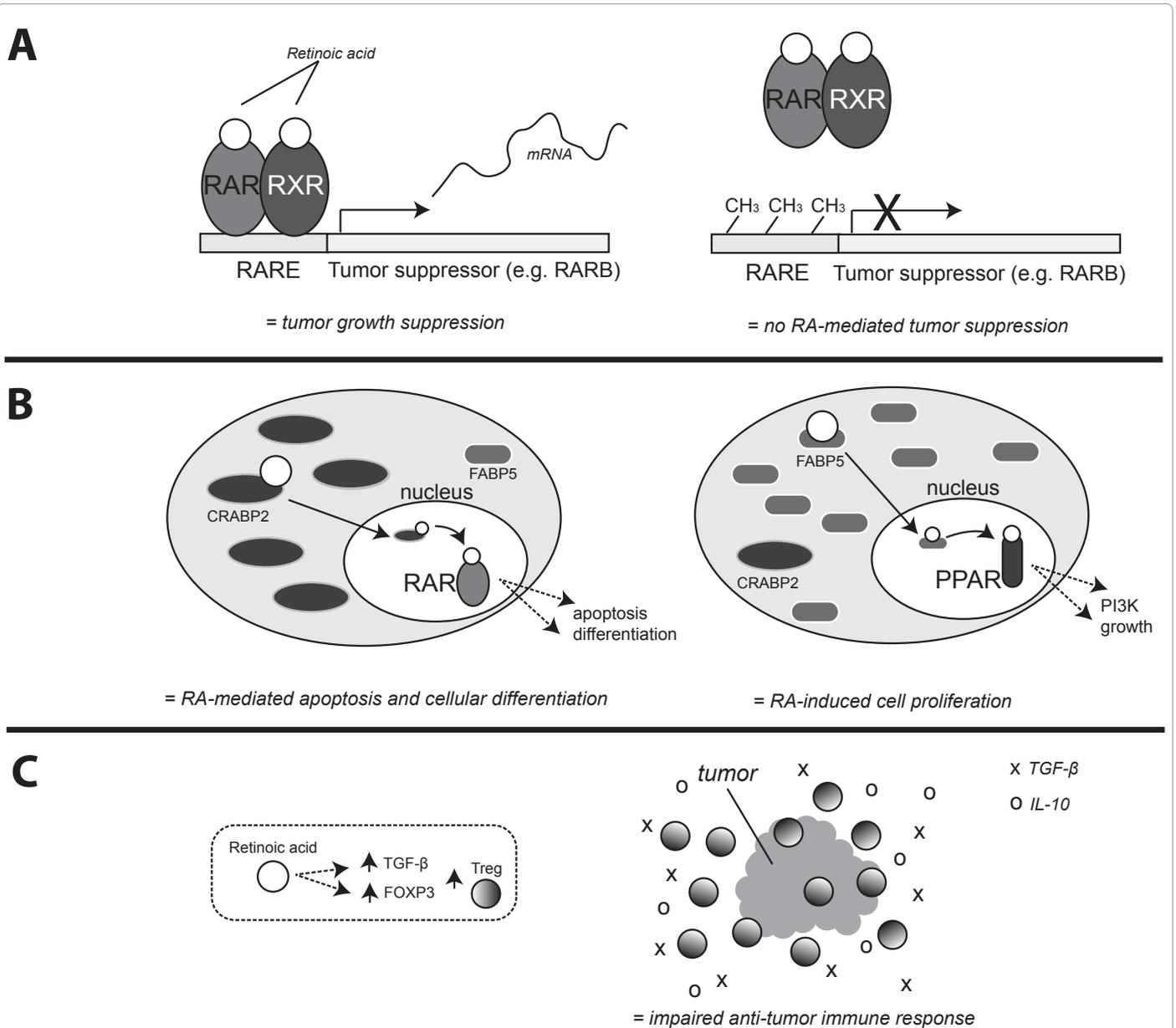
In addition to genes with RAREs, RA can also directly regulate expression of genes without RAREs by interacting with other NHRs. The interactions between RA and peroxisome proliferator-activated receptors (PPARs) and activator protein 1 (AP1) transcriptional activation have been implicated in cancer. As well, there is evidence to support cross-talk between the estrogen and RA signaling pathways.

### Peroxisome proliferator-activated receptor

In the absence of abundant CRABP2 in the cytoplasm (Figure 2), RA can bind other chaperones such as fatty-acid binding protein 5 (FABP5) with decreased affinity [9,95,96]. Binding to FABP5 delivers RA to PPAR $\beta$ / $\delta$  isoforms instead of to RARs (Figure 3B) [95,97,98]. PPAR $\beta$ / $\delta$  heterodimerize with RXRs and bind to peroxisome proliferator response elements (PPREs, a DR1 of the HRE, AGGTCA), leading to the transcription of genes typically involved in cell growth, such as 3-phosphoinositide-dependent protein kinase 1 (PDK1) [99-102]. Therefore, predominant RA signaling via FABP5/PPAR induces cell proliferation (Figure 3B) as opposed to the more typical cell cycle arrest induced by CRABP2/RAR signaling [98]. This hypothesis has found clinical significance in triple-negative breast cancer, pancreatic cancer and glioblastoma [98,103-107], and it may also find significance in head-and-neck squamous cell carcinomas (HNSCCs) [108].

### Activator protein 1

It is well documented that RARs antagonize the action of the AP1 transcription factor [109,110]. Transrepression of AP1 by RA is possibly



**Figure 3:** Potential mechanisms for retinoic-acid-mediated tumor suppression or progression. RA is involved in transcription of several tumor suppressor genes such as RARB and RARRES1; when the regulatory regions of these genes are epigenetically modified by DNA methylation or histone deacetylation, transcription is reduced and RA is unable to mediate these suppressive effects (A). In addition, the ratio of cellular CRABP2:FABP5 is indicated in promoting opposite cellular effects. In the presence of high CRABP2, RA activates RARs which canonically leads to apoptosis and differentiation; however, in the presence of high FABP5, RA activates PPAR isoforms which leads to PI3K-mediated cellular proliferation (B). A peripheral mechanism by which RA mediates tumor progression is via the induction of immunosuppressive Tregs (C). The presence of high numbers of Tregs leads to impaired anti-tumor immunity and immune escape; the secretion of TGF- $\beta$  as well as IL-10 mediate further immunosuppressive effects.

coordinated by the interaction of the RAR $\alpha$  DNA-binding domain with the c-Jun portion of the AP1 heterodimer [111], which leads to a decrease in RNA polymerase II recruitment [112]. AP1-dependent transcription regulates the expression of oncogenic proteins and those involved in proliferation, such as metalloproteases, VEGF and TGF- $\beta$ . Thus, RAR-mediated antagonism of AP-1 has been implicated in the antiproliferative effects of RA in gastric cancer, breast cancer, ovarian cancer as well as cervical cancer cells [109,110,113-115].

### Estrogen receptor

RAR $\alpha$  expression is induced by estrogen [116,117]; as well, there is

evidence for extensive cross-talk between RA/estrogen transcriptional activities. SMRT, an RAR/RXR corepressor, is recruited to estrogen receptor alpha (ER $\alpha$ ) in the presence of estradiol and its expression is required for full transcriptional activity of ER responsive genes [118].

Recently, Hua *et al.* documented that RAR binding in MCF-7 breast cancer cells was extensively collocated with ER $\alpha$  binding, which most often results in competitive binding and antagonism between the two NHR signaling pathways [119]. Similarly, it has been shown that RA inhibited ER-mediated transcription of epidermal growth factor receptor (EGFR) by competing for the same binding site [120].

Additional antagonism between the two signaling pathways has been demonstrated in the regulation of several microRNAs [121].

In contrast, Ross-Innes *et al.* found that RAR $\alpha$  is a requirement for ER transcription and that RAR $\alpha$  and ER $\alpha$  co-occupation of the same regulatory regions is cooperative and leads to enhanced gene transcription; however, atRA inhibited the ER-RAR interaction [122]. Clinically, RAR $\alpha$  has been associated with tamoxifen resistance in two independent breast cancer patient cohorts, which suggests that RAR $\alpha$  may be required to maintain coactivators for ER-mediated transcription [123].

Regardless of the nature of their interactions (cooperative or competitive), it seems evident that cross-talk between ER- and RAR-induced signaling pathways needs to be examined when hormonal therapies are recommended. As well, both pathways should be considered when first-line ER antagonists (e.g. tamoxifen) fail or induce resistance.

### Retinoic acid induction of apoptosis in cancer

Dysregulated apoptosis is a major hallmark of cancer, and the RA pathway contributes to apoptosis by various mechanisms. Furthermore, cancer cells vary greatly in their sensitivity to atRA-induced cytotoxicity, with RA regulation of apoptotic pathways contributing to this differential growth suppressive response. For example, nM quantities of RA induce cell death in MCF7 and T47D breast cancer cells, while in MDA-MB-231 breast cancer cells, even  $\mu$ M quantities are insufficient [124-126]. Although the interaction of atRA with apoptosis pathways is not the only explanation of RA sensitivity or resistance, it is a factor to be considered.

The most obvious role for RA in apoptotic regulation is via regulation of gene expression of apoptosis mediators and effectors. RA has been shown to downregulate several anti-apoptotic proteins including B-cell lymphoma 2 (BCL2) and survivin [127,128]. While AP1 transrepression is also implicated in downregulation of BCL2 signaling, the authors note that this did not fully account for the antiproliferative effect observed; this suggests that other RA responsive genes may be partially responsible for inducing apoptosis in breast cancer MCF-7 and ZR-75 cells [128]. Furthermore, RA upregulates the tumor suppressor p53, a major player in apoptosis; however, the mechanism for this induction is not fully understood. STRA6 has been implicated as well as canonical ligand-RAR transcriptional activation [129-131]. It is possible that epigenetic repression via methylation of the p53 promoter may confer resistance to RA-mediated growth inhibition. There may also be a role for RA mediating resistance to p53-dependent apoptosis, as has been demonstrated in SH-SY5Y neuroblastoma cells [132].

RA also plays a role in the TNF-related apoptosis-inducing ligand (TRAIL) and Fas extrinsic apoptotic pathways. TRAIL-induced apoptosis is mediated by the binding of the TRAIL cytokine to its receptors TRAILR1 (or death receptor DR4) and TRAILR2 (DR5). It appears that cancerous cells are more sensitive to TRAIL-mediated apoptosis [133,134]. RAR $\alpha$ -specific ligands and RA were shown to induce apoptosis via TRAIL in NB4 APL cells and SK-BR-3 breast cancer cells; this effect is mediated by RA induction of interferon regulator factor 1 (IRF-1) [135,136]. Similarly, RA upregulated expression of TRAILR1 on NSCLC and HNSCC cells and enhanced apoptosis [137].

Apoptosis via the Fas pathway occurs when Fas ligand (FasL) binds to and trimerizes its receptor Fas on cell membranes. Fas is

often downregulated in cancers, or a decoy receptor can be expressed which binds to FasL and prevents FasL-Fas interactions [138-143]. A number of studies indicate that RA represses expression of FasL [144-148]. Interestingly, Toth *et al.* found in 2004 that atRA and 9-cis-RA were able to induce FasL expression, but only RAR $\gamma$ -specific agonists were able to induce apoptosis in IP-12-7 T hybridoma cells [149]. The authors suggest that ligand binding to RAR $\alpha$ /RAR $\beta$  sensitizes the Fas receptor cell-death pathway.

Furthermore, there are also instances where elements of the RA pathway affect apoptosis without involvement of RARs/RXRs and transcriptional regulation. For example, RA chaperone CRABP2 has been implicated in caspase 9 (CASP9) signaling in an RA-independent mechanism [150]. While RA induced the expression of CASP9, overexpression of CRABP2 in the absence of RA induced apoptotic protease-activating factor-1 (APAF1) expression and cleavage of CASP9 [150]. CASP9 can then further initiate apoptotic signaling by cleaving several other pro-caspases to their active forms.

The mechanisms by which RA contributes to the apoptotic pathways are varied, and the responses of different cell types depend on the status of the apoptotic machinery within the cell. Silencing (via epigenetics or mutation) of apoptotic pathway components affects the response to RA treatment and therefore are also determinants in the involvement of the RA pathway in cancer and the application of retinoids as cancer treatments.

### Tumor immunity and retinoic acid

Vitamin A deficiency is associated with defects in immune function [151,152]; supplementation seems to restore function [153]. It stands to reason that dysregulation of RA signaling in cancers could also be associated with malfunctioning immunity.

For a cancer to develop and progress, it has to continually evade immunosurveillance and escape targeting and destruction by the host immune system. Cancers have several immune evasion mechanisms; for instance, tumor cells have decreased antigen presentation by down-regulation of antigen-processing machinery such as transporter associated with antigen processing 1 (TAP1) and TAP2, as well as low molecular mass protein 2 (LMP2) and LMP7 which are associated with the major histocompatibility complexes (MHCs) [154-156]. Other mechanisms include induction of immunosuppression via T-cell anergy, generation of regulatory T-cells, or secretion of immunosuppressive cytokines such as interleukin-10 (IL-10) and TGF- $\beta$  [157]. As discussed below, RA plays a critical role in these immune processes with both anti-tumor and cancer-promoting consequences.

### Antigen processing

A variety of evidence in different model systems suggests that RA up regulates MHC components and enhances antigen processing and presentation. In RA-mediated differentiation of human embryonic stem cells to embryoid bodies, Suarez-Alvarez *et al.* noted upregulation of MHC components and molecules associated with antigen processing such as TAP1, TAP2 and LMP7 [158]. RA treatment of human embryonal carcinoma and human cervical cancer cells induced MHC I expression [159,160]. Similarly, retinoid treatment of neuroblastoma cell lines increased MHC I expression and sensitized those cells as well as uveal melanoma cells to killing by CD8<sup>+</sup> cytotoxic lymphocytes (CTLs) [161,162]. Modulation of antigen processing and presentation by RA may contribute to the anti-tumor effects seen in some cancers.

## T-cell activation and anergy

T-cell activation requires antigen activation in combination with a co-stimulatory signal. If antigen is presented to a T cell but CD28 does not bind to B7.1/7.2 (CD80/86), the T-cell undergoes anergy. There is conflicting evidence on the modulation of CD80/CD86 expression by RA. Zhan *et al.* found that atRA downregulated CD80 and CD86 on dendritic cells in an experimental autoimmune encephalomyelitis model [163]; however, RA has been shown to induce CD80 on murine splenic mononuclear cells following tetanus immunization as well as CD80/CD86 expression on neuroblastoma cells [164] (and unpublished data, reviewed in [165]).

## Tumor-specific T-cells

It has been shown that RA can improve specific CD8+ T-cell responses to vaccines [166]. Guo *et al.* have recently established that increased RA production within a tumor is necessary for CD8+ T-cell expansion and accumulation [167]. It is becoming increasingly apparent that RA plays an important role in CD8+ T-cell function; however, the precise effects on effector cells as compared to memory cells remain to be determined [168]. Further insight into the mechanisms by which RA can enhance CD8+ T-cell responses will be of benefit and may allow targeted immunomodulatory therapy via retinoid treatment.

## Regulatory T-cells

The majority of T lymphocytes develop into CD8+ CTLs or CD4+ Th1/Th2 cells which are responsible for key adaptive immune functions. A smaller population of CD4+ cells also express CD25 and cytotoxic T-cell antigen-4 (CTLA-4) as well as forkhead box P3 (FoxP3); this subset, known as regulatory T (Treg) cells [169,170], play a critical role in the tumor immunosuppressive response. This response is essential for cancer progression.

The natural role of Tregs is to prevent auto-immune reactions when the immune system mounts a strong response to a pathogen. Tregs produce IL-10 which further inhibits T-cell proliferation [171,172]. Additionally, TGF- $\beta$  plays a role in Treg development from CD4+ T cells [173,174]; and this can be promoted by RA via several mechanisms including epigenetic regulation of the Foxp3 promoter (Figure 3C) [175-177]. Additionally, RA induces TGF- $\beta$  expression [178,179] and TGF- $\beta$  can modulate the expression of RAR $\beta$  [180], possibly generating a feedback loop. These findings suggest that induction of tolerance by increased RA signaling within some cancers (Figure 3C) may be an important target for anti-cancer immunotherapy [181].

Due to the various functions of the immune system mediated by RA, it is likely that the direction of the immune modulation (pro-inflammatory or immune escape) is dependent on the cellular context.

## Retinoids in Cancer Prevention and Therapy

### Acute promyelocytic leukemia

To date, the most successful use of RA in cancer therapy has been in the treatment of APL. APL represents approximately five to eight percent of all acute myeloid leukemia cases and is considered very severe. The vast majority (98%) of APL patients present with a chromosomal rearrangement and fusion of the promyelocytic leukemia (PML) and RAR $\alpha$  genes [182,183]. This fusion delays apoptosis [184]; this is likely due to the inability of the PML-RAR $\alpha$  fusion protein to appropriately induce physiological RA-mediated differentiation and apoptotic pathways as a result of enhanced recruitment of co-repressor complexes [185,186]. These complexes and their accompanying HDAC

activity prevent induction of RA-responsive genes at physiological RA concentrations [185]. This causes an accumulation of granulocyte precursors, promyelocytes. Treatment of APL with supraphysiological concentrations of atRA induces terminal differentiation of these cells and significantly improves patient outcomes [187]. Most recently, atRA in combination with arsenic trioxide (As<sub>2</sub>O<sub>3</sub>), a second-line drug in the treatment of leukemias, has been used in treatment of APL by several groups with improved efficacy [188-190]. The atRA/As<sub>2</sub>O<sub>3</sub> combination decreased time to achieve complete remission [188], and increased event-free and overall survival over treatment with atRA alone [190]. These studies suggest that atRA and As<sub>2</sub>O<sub>3</sub> work synergistically to enhance apoptosis and differentiation [191]. The success of atRA in treating APL has led to earnest attempts to use retinoids in the treatment of other cancers. Unfortunately, as discussed below, the results on several of the most prevalent cancers and those with high mortality have been mixed at best.

### Breast cancer

RA is highly effective in suppressing the growth of many cultured breast cancer cell lines, although some cell lines (e.g. MDA-MB-231) are highly resistant and proliferation may even be stimulated when cultured with atRA [124-126,192-201]. Regardless, the general anti-proliferative effects of atRA observed *in vitro* have resulted in a number of clinical studies investigating the use of retinoids in treating breast cancer.

Combination therapy of atRA with the chemotherapeutic agent paclitaxel has been tested in breast cancer patients with recurrent or metastatic disease. Unfortunately, the combination treatment gave modest results, with no improvement over paclitaxel alone in disease progression and survival; however, increased stable disease was observed and was attributed to atRA's known role in inducing cell cycle arrest and differentiation [202].

Fenretinide, a retinol analogue (Figure 1B), has also been investigated as a preventative agent as it is associated with fewer adverse events than the biological retinoid derivatives [203]. One clinical trial administered fenretinide orally over five years. At the five-year end point, there was no significant difference in breast cancer recurrence in patients treated with fenretinide [204]. A fifteen-year follow-up of less than half of the original patients found a 30% decrease in recurrence of breast cancer among premenopausal women treated with fenretinide [205]. A number of criticisms have been directed at these studies, including the use of post-hoc analyses [206] as well as the exclusion of hormone receptor status from the data collected [207]. The conclusions of this study thus require further investigation before fenretinide is adopted as a chemopreventative agent. Thus far, the indications are not promising, as a clinical trial of fenretinide and tamoxifen in prevention of breast cancer among high-risk premenopausal women revealed no effect of combination therapy when compared to placebo treatment, despite both components individually reducing breast cancer risk [208]. We await the results of the long-term fenretinide treatment currently being investigated in young women at increased risk for breast cancer (NCT01479192) to reveal if retinoids may yet prove beneficial in the treatment of certain breast cancers or in breast cancer prevention as tamoxifen alone achieves limited success in increasing survival of patients with ER+ tumors [209,210].

Rexinoids are less toxic than their RAR-specific counterparts; however, the RXRs are responsible for eliciting pleiotropic cellular effects as they can heterodimerize with a number of NHRs. As such, the many possible effects and pathways RXR could interfere with

limited the options for rexinoid modulation of cancer [186]. As the regulation of RXR signaling is elucidated, rexinoid treatment may become more refined, decreasing the chance of undesired pan-cellular effects. In an MMTV-neu transgenic mouse model of ER<sup>+</sup> breast cancer, treatment with the RXR-specific ligand rexinoid LG100268 (Figure 1C) in combination with the anti-estrogen acolbifene prevented tumor formation. When tumors were allowed to form, rexinoid/acolbifene therapy caused tumor regression. Acolbifene treatment alone did not induce regression [211]. This data supports the consideration of hormone antagonist therapy in combination with rexinoids in hormone-receptor-negative tumors.

### Ovarian cancer

As in breast cancer, there is evidence to support abnormal retinoid signaling in ovarian cancers. Patients with ovarian tumors have decreased concentrations of retinol [212], as well as RBP [212,213]. Increased vitamin A and carotene intakes have been associated with a reduced risk of ovarian cancer, particularly among smokers [214]. As well, promising pre-clinical data such as the increased survival of tumor-bearing mice following intracavitary treatment of ovarian carcinoma xenografts with fenretinide has led to clinical trials with retinoids [215].

In the previously mentioned 5-year clinical trial using fenretinide to prevent secondary malignancies in women with breast cancer, significantly fewer women developed ovarian cancer during the 5-year treatment period [204]. The patients who developed ovarian cancer did so after treatment cessation [204,216,217]. This suggests that fenretinide may exhibit a preventative effect on ovarian tumors, but that this effect does not persist after treatment is discontinued. A more recent trial of post-chemotherapy IL-2 and 13-cis-RA (isotretinoin, Figure 1A) combination treatment in patients with advanced ovarian cancer improved progression-free and overall survival [218]; this is likely due to improved immunological parameters [218-220].

In contrast, other studies have given less promising results. A phase I-II biomarker trial on fenretinide in ovarian cancer revealed no biological effect; however, this may be due to the inability to accumulate therapeutically active concentrations of fenretinide [221]. Treatment of asymptomatic, post-chemotherapy patients with ovarian cancer with 13-cis-RA and calcitriol (the hormonally active form of vitamin D) revealed no effect on tumor progression as measured by cancer antigen-125 (CA-125) levels [222]. CA-125 (or MUC16) is a cancer antigen commonly used as a biomarker for ovarian cancer; increasing CA-125 levels following complete remission is strongly indicative of recurrent disease [223].

### Pancreatic cancer

Pancreatic cancer is typically diagnosed at advanced stages and a majority of patients present with metastatic disease [224,225]. Treatments targeting novel signaling pathways may therefore be beneficial to patients who do not respond to conventional therapies. There is substantial evidence to suggest that the RA signaling pathway is important in pancreatic cancer. A number of murine pancreatic cancer models (chemically and genetically induced) displayed significantly reduced retinoid signaling [226]. Early clinical trials demonstrated low toxicity of 13-cis-RA in combination with IFN- $\alpha$  [227,228]; however, no objective response was observed [228]. Gemcitabine is a common therapeutic agent for many cancers, including pancreatic cancer. In a 2007-reported pilot phase II study, a combination of 13-cis-RA with gemcitabine was well tolerated, but did not improve response rate

among patients with unresectable pancreatic cancer [229].

In light of recent findings regarding the FABP5:CRABP2 ratio as discussed above, Gupta *et al.* profiled fourteen patient-derived pancreatic cancer lines and propagated them as xenografts in athymic nude mice with atRA treatment. They demonstrate that a low FABP5:CRABP2 dictates response to atRA and suggest that atRA be reconsidered in treatment of pancreatic cancer for those patients with FABP5-negative tumors; or, in combination with re-expression of endogenous CRABP2 [103]. This finding may yet explain the inefficacy of 13-cis-RA in previous trials for pancreatic cancer, where patients were not screened for FABP5 and CRABP2 levels.

Alternatively, artificial retinoids may prove to be more effective in therapy for pancreatic cancer than the natural retinoic acid isoforms. *In vitro* data on acyclic retinoid (Figure 1B) in combination with gemcitabine suggests that this combination therapy induces apoptosis and inhibits cell proliferation in pancreatic cancer cell line Panc-1 [230]. Interestingly, acyclic retinoid has decreased recurrent and second primary tumors in clinical trials for hepatocellular carcinoma [231].

### Lung cancer

Despite evidence in the late 1990s that supplementation of  $\beta$ -carotene with retinyl ester among workers at high risk showed adverse effects on the incidence of lung cancer among patients [232], and a similar study of  $\beta$ -carotene supplementation which revealed no effect on lung cancer incidence [233], retinoids continue to be studied in prevention and treatment of lung cancer. For example, a phase II clinical trial indicated that the addition of atRA to a cisplatin/paclitaxel chemotherapy regimen increased the response rate as well as overall survival time in patients with NSCLC [234].

A 2011 meta-analysis of evidence pertaining to the use of retinoids in lung cancer concluded that there is little support for the use of vitamin A and its natural derivatives in the treatment and prevention of lung cancer; however, bexarotene (Figure 1C) may hold promise for some patients [235]. One 2001 study observed better-than-expected survival among NSCLC patients treated with bexarotene in combination with cisplatin and vinorelbine [236]. A phase III trial of bexarotene in combination with cisplatin and vinorelbine in NSCLC revealed a benefit only to patients whom developed high-grade hypertriglyceridemia [237]. Similar results were observed in a phase III trial using bexarotene in combination with carboplatin and paclitaxel [238].

A recently completed clinical trial has investigated the addition of interferon alpha (IFN $\alpha$ ) and 13-cis-RA to paclitaxel treatment of recurrent small cell lung cancer (NCT00062010). The premise of this phase II study was to reduce resistance to paclitaxel treatment by modulating expression of BCL2. The full results of this trial have not yet been published.

As discussed earlier, there is increasing evidence for RA signaling via the tumor stroma. Therapeutically, the targeting of this signaling environment may have some promise, as 20% of smokers at-risk for lung cancer treated with 13-cis-RA exhibited a restoration of normal RAR $\beta$  expression in the bronchial epithelium [239]. The variance observed in expression of components of the RA signaling pathway in the tumor stroma may also contribute to variable responses of patients to retinoid therapy.

## Prostate cancer

There is evidence for aberrant retinoid signaling in prostate cancers as well. Prostate carcinoma tissues contain significantly less RA than normal prostate or benign prostate hyperplasia tissues [240]. Retinoids have been tested in clinical trials for prostate cancer since the 1990s. Reports from clinical trials using atRA for hormone-refractory prostate cancer revealed no or minimal response among patients [241,242]; this may be related to enhanced clearance of atRA [241]. Similarly, fenretinide, which has been used successfully to inhibit xenograft growth of prostate MLL cells in rats [243] was overall ineffective in clinical trials. In a phase II fenretinide clinical trial, patients with rising prostate-specific antigen (PSA) showed no significant decrease in PSA over the course of treatment [244]. In patients with confirmed prostate cancer prior to radical prostatectomy, a 3-week course of fenretinide was used to evaluate prostate cancer biomarkers. No preventative effect on chemopreventative biomarkers was observed. It is possible that with the development of new formulations of fenretinide (as discussed below for neuroblastoma) increased efficacy may be observed.

Alternatively, the potential use of retinoids in combination therapies may be a more effective treatment option for prostate cancer. For example, 13-cis-RA in combination with IFN $\alpha$  and paclitaxel was well tolerated and decreased paclitaxel clearance in patients [245]. This treatment may function synergistically to inhibit BCL2 anti-apoptotic activity in prostate cancers.

## Neuroblastoma

For neuroblastoma, the most common extracranial solid tumor of children, treatment efficacy with retinoids has also been mixed. Treatment with myeloablative chemotherapy, total-body irradiation and autologous bone marrow transplant followed by 13-cis-RA treatment in pediatric patients with high-risk neuroblastoma increased the 3-year event-free survival over treatment without 13-cis-RA [246]. A long-term follow-up of those patients revealed significantly improved overall survival when treated with 13-cis-RA [247]. In contrast, in 28 children with advanced neuroblastoma that was refractory to conventional therapy, 13-cis-RA treatment failed to ameliorate their condition [248]. Studies with fenretinide have generally been less promising. A phase II study of oral capsular fenretinide found limited efficacy among patients with refractory or recurrent neuroblastoma; this was likely due to low bioavailability of this particular formulation of fenretinide [249]. New clinical trials with improved formulations (NCT00295919, NCT00646230) are ongoing [250]. Additionally, atRA was investigated in combination with IFN $\alpha$ 2a for treatment of pediatric neuroblastoma or Wilms' tumor and found to be ineffective [251].

## Conclusions

Impaired RA signaling in cancer remains an interesting target in prevention, disease progression, and therapy. In particular, the highly effective combination of atRA with As<sub>2</sub>O<sub>3</sub> in treating APL fuels continued attention to retinoid-based therapy. Unfortunately, the wide variability in RA signaling potential and downstream effects prevents the global applicability of RA to cancer prevention and anti-cancer therapy (Figure 3). Strikingly, data suggesting the benefits of  $\beta$ -carotene supplementation on chemically-induced carcinogenesis *in vivo* [252] was not supported by later data in spontaneous carcinogenesis [253], or in clinical trials [232,233]. This suggests that retinoids may only be able to exert preventative effects when oncogenic events have already been initiated.

Natural retinoids have been associated with high toxicities and

low efficacies [252]; this led to the development of modified, artificial retinoids. Synthetic retinoid analogues such as fenretinide and the RXR-selective bexarotene have shown mild successes in cancer treatment; this may improve with better drug delivery systems. From a review of the literature, it is clear that the pleotropic effects of RA are cellular-context dependent and vary greatly. Potential factors for consideration include epigenetic modifications, accessory signaling factor expression, and cross-talk with other signaling pathways. Identifying the variables in the RA signaling pathway which determine retinoid response (e.g. cell cycle arrest or proliferation, Figures 3A and 3B) is necessary if retinoid-based therapies are to become more widely utilized in the treatment of cancer. The stratification of patients into clusters based on expression of RA-inducible genes as well as genes involved in other, intertwined signaling pathways will see increased efficacy in select patient groups. Ongoing research into the role of RA signaling in cancer cells, anti-tumor immune response, and the tumor microenvironment will allow increased personalization of therapeutic regimens as our improved understanding of this pathway may permit better predictions of response rates.

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