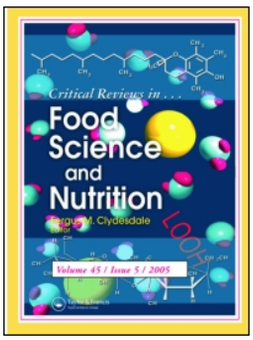

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Vitamin A and the epigenome

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ABSTRACT

The epigenetic phenomena refer to heritable changes in gene expression other than those in the DNA sequence, such as DNA methylation and histone modifications. Major research progress in the last few years has provided further proof that environmental factors, including diet and nutrition, can influence physiologic and pathologic processes through epigenetic alterations, which in turn influence gene expression. This influence is termed nutritional epigenetics, and one prominent example is the regulation of gene transcription by vitamin A through interaction to its nuclear receptor.

Vitamin A is critical throughout life. Together with its derivatives, it regulates diverse processes including reproduction, embryogenesis, vision, growth, cellular differentiation and proliferation, maintenance of epithelial cellular integrity and immune function.

Here we review the epigenetic role of vitamin A in cancer, stem cells differentiation, proliferation, and immunity. The data presented here show that retinoic acid is a potent agent capable of inducing alterations in epigenetic modifications that produce various effects on the phenotype. *Medical benefits of vitamin A as an epigenetic modulator, especially with respect to its chronic use as nutritional supplement, should rely on our further understanding of its epigenetic effects during health and disease, as well as through different generations.*

KEYWORDS

Vitamin A; nutrition; nutritional epigenetics; epigenetics

Introduction

The function of genes is determined by the epigenetic phenomena and not always by the DNA sequence and code, referring to the inheritance of traits through mechanisms that are independent of DNA primary sequence, and includes the inheritance of gene expression patterns and/or expression levels that contribute to phenotypic differences among individuals (Gravina and Vijg, 2010; Skinner, 2011). It is a difference in the organisms' phenotypes that occur without any accompanying changes or mutations in their genotypes. DNA methylation, histone modification, and synthesis of noncoding microRNAs are the three main epigenetic mechanisms regulating gene expression (Mohr et al., 2011; Niculescu and Lupu, 2011; Skinner, 2011). These epigenetic alterations may be caused, among other things, by nutritional factors.

Nutrition is one of the primary environmental exposures that determine health. During our lifetime, nutrients can modify physiologic and pathologic processes through epigenetic mechanisms that are critical for gene expression (Gluckman et al., 2011; Niculescu, 2012). Dietary and lifestyle factors can interact with genes and may have a negative impact on health that can predispose for the development of cardiovascular diseases, type 2 diabetes and cancer, while other nutrients can have an opposite, protective effect. This effect of nutrients, involving DNA/chromatin modulations which programs or reprograms biological pathways with multigenerational consequences, is termed *nutritional epigenetics*. One prominent example is the regulation of gene transcription by vitamin A through interaction to its nuclear receptor.

Vitamin A is a generic term for a large number of related compounds. Retinol (an alcohol) and retinal (an aldehyde) are often referred to as preformed vitamin A. Retinal can be converted by the body to retinoic acid (RA), the form of vitamin A known to affect gene transcription. Retinol, retinal, retinoic acid, and related compounds are known as retinoids. Beta-carotene and other carotenoids that can be converted by the body into retinol are referred to as provitamin A carotenoids (Theodosiou et al., 2010) (Figure 1).

The metabolism and actions of vitamin A and the carotenoids have been extensively studied. Vitamin A and its derivatives are required throughout life and regulate diverse processes including reproduction, embryogenesis, vision, growth, cellular differentiation, and proliferation, maintenance of epithelial cellular integrity and immune function (De Luca, 1991; Duester, 2008; Mark et al., 2006; Stephensen, 2001). The ability to target and affect multiple pathways, as well as its small, tightly-controlled production, attributes hormone-like actions to the principal metabolite of vitamin A- RA (Ross and Ternus, 1993). In fact, several biochemical and molecular studies have shown that the mechanism of action of RA is highly analogous to that of the steroid hormones—estrogens, progestins, glucocorticoids, 1,25-dihydroxyvitamin D—and thyroid hormone, which usually act by gene activation/deactivation. These hormones, much like RA, are transported through the circulation in

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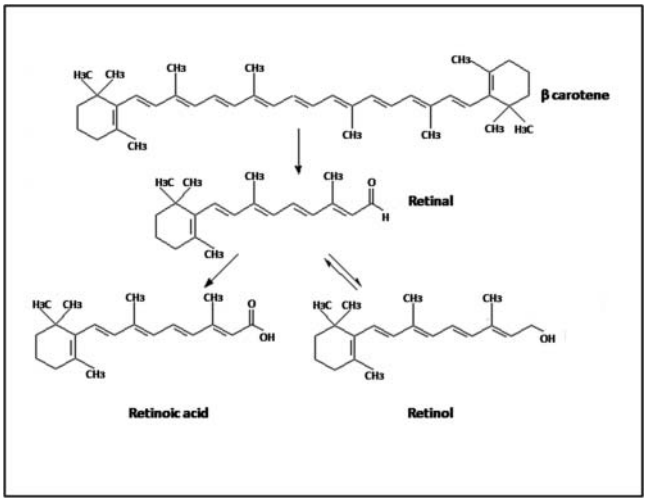

Figure 1. Chemical structures of vitamin A main derivatives.

association with a hormone−binding protein and are soluble in the plasma membrane of the cell. Their receptors are intracellular, and they act on gene transcription rather than at the translation level. Thus, they act more slowly than do the soluble hormones, on the scale of days rather than minutes (Brinkmann, 1994).

When signaling in a paracrine fashion, RA must be released from the cytoplasm and taken up by receiving cells; however, RA can also act in an autocrine manner. RA enters the nucleus, assisted by CRABP2 (cellular retinoic acid binding protein 2) (Budhu and Noy, 2002), and binds to a transcription complex which includes a pair of ligand-activated transcription factors comprising the RA receptor (RAR)–retinoic X receptor (RXR) heterodimer, targeting RA responsive elements (RAREs) within specific gene promoters and enhancers (Maden, 2007).

In light of the increasing information in the literature discussing epigenetic traits following RA treatment, we review here, the epigenetic role of vitamin A in areas of interest where vitamin A is potentially of influence: in stem cells, cancer, and immunity.

The main acceptable and well-established epigenetic mechanisms relevant to nutrition and/or vitamin A include: 1. DNA methylation, which is the covalent addition of a methyl (CH_3) group at the 5-carbon of the cytosine ring located next to a guanidine nucleotide (CpG dinucleutides) (Cucu, 2011; Miyamoto and Ushijima, 2005). In normal cells, CpG islands in the promoter regions of expressed genes are usually unmethylated, whereas CpGs elsewhere in the genome are usually methylated (Teodoridis et al., 2004). DNA methyltransferases (DNMTs) control DNA methylation of CpG residues and its maintenance. 2.Histone modifications, that include methylation, acetylation, ubiquitination, phosphorylation, sumoylation, ADP ribosylation, biotinylation, and proline isomerization (Hake, 2011). Core histone molecules are organized in octamers (consisting of H2A, H2B, H3, and H4, two of each) that form the complex nucleosome structure. Specific amino acids of histone tails are targets for the above mentioned posttranslational modifications. For example, histone acetylation increases their net negative charge that in turn interrupts their interaction with DNA, leading to open chromatin structure, which allows initiation of gene transcription and expression. On the other hand, when histone deacetylases (HDACs) are recruited, positive charges are introduced in the histone tail, which tightly bind to negatively charged nucleic acids, leading to the formation of compact chromatin structure that prevent transcription. The third epigenetic mechanism (although still controversial) is miRNAs which are capable of regulating DNA methylation and histone modifications and thus controlling the expression of a cohort of cognate target genes (Sato et al., 2011).

Generally, DNA hypomethylation, histone acetylation and histone H3K4 methylation have been associated with active chromatin, whereas DNA hypermethylation, histone deacetylation, and histone H3K9 and K27 di- or trimethylation have been found in inactive chromatin regions (Strahl and Allis, 2000).

Vitamin A and cell differentiation and proliferation

Vitamin A and stem cells

RA plays an important role in mediating the growth and differentiation of both normal and transformed cells (Connolly et al., 2013; Duester, 2008).

"In vitro", RA induces differentiation of human embryonic stem cells (hESCs) into a number of specific cell types (Gudas and Wagner, 2011). Differentiation of a specific cell type involves the establishment of a precise epigenetic profile composed of genome wide epigenetic modifications such as DNA methylation and histone modification (Cheong et al., 2010). When DNA methylation and gene expression assays were utilized to generate whole-genomic methylation and gene expression profiles for both undifferentiated hESCs and RA-treated hESCs (Cheong et al., 2010), it was found that the average methylation level in the RA-treated hESCs (29.5%) was greater than in the undifferentiated hESCs (27.1%). For example, CpGs on the CHFR gene (checkpoint with forkhead associated and ring finger) were among the most hypermethylated genes after RA treatment. Methylation of CHFR is known to be associated with silencing of CHFR expression in various types of cancer (Toyota et al., 2003), and CHFR is also known as a tumor suppressor (Yu et al., 2005). This means that cells in which CHFR was epigenetically inactivated constituted differentiated hESCs (Cheong et al., 2010). When comparing the DNA methylation patterns between undifferentiated hESCs and RA-treated hESCs, the researchers found that most genes hypermethylated after RA treatment fit in the standard paradigm of extensive methylation being correlated with gene silencing. For instance, THY1, which plays a critical role in maintaining the undifferentiated status of embryonic stem cells, was hypermethylated and downregulated in response to RA treatment (Cheong et al., 2010).

Another epigenetic mechanism influenced by RA is histone modification. Several studies show that upon RA exposure at RAR-regulated genes, the H3K27me3 repressive marks decrease while the H3K4/9ac activation marks increase, allowing for transcriptional activation of target gene expression that regulate stem cell differentiation (Amat and Gudas, 2011; Gillespie and Gudas, 2007; Kashyap and Gudas, 2010). For example, using chromatin immunoprecipitation experiments

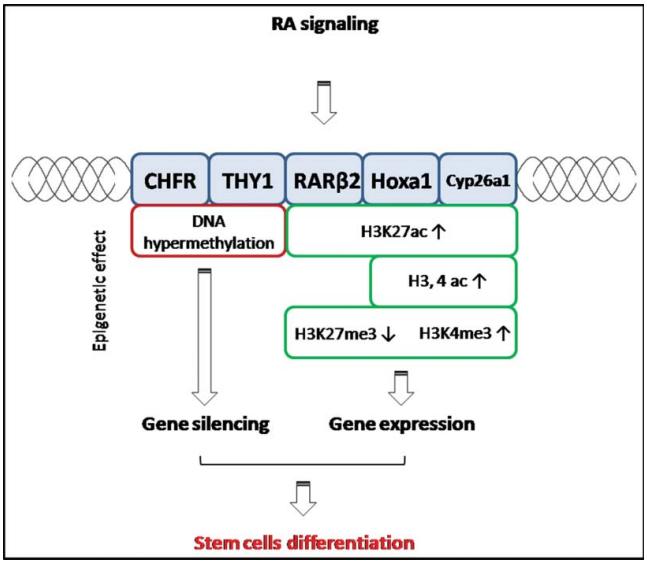

Figure 2. RA-mediated epigenetic changes in genes involving stem cells differentiation. Relevant genes are presented in blue rectangles. Epigenetic effects in green and red represent histone modifications and DNA methylation, respectively.

in embryonic stem cells, it was found that RA treatment differentially mediated the removal of HDACs from Hoxa1, Cyp26a1, and RARβ2 genes, promoting the deposition of the H3K27ac mark at these genes, thereby promoting stem cells differentiation (Urvalek and Gudas, 2014).

Epigenetic changes in the genes mentioned above, involving stem cells differentiation and proliferation, is presented in Figure 2.

Vitamin A and cancer

Cancer initiation and progression are driven by the concurrent changes in the expression of multiple genes that occur via genetic and epigenetic alterations, leading to either activation of oncogenes and prometastatic genes, or silencing of tumor suppressor genes and to genome rearrangements and instability (Stefanska et al., 2012; Szyf, 2005; Widschwendter and Jones, 2002). Epigenetic modifications have attracted a significant amount of attention in the prevention and treatment of different illnesses, with cancer at the forefront, mainly due to their reversibility.

Induction of differentiation is considered to be therapeutically advantageous in cancer situations, and differentiating agents, including RA, have become standard clinical treatment (Mongan and Gudas, 2007; Reynolds et al., 2003). For example, exogenous retinoids have been shown to suppress precancerous lesions and prevent the development of secondary cancers (Hong et al., 1994; Pastorino et al., 1993). The potential for induction of differentiation makes RA signaling a promising molecular target for neuroblastic tumors, where differentiation is a key feature in pathologic classification and prognosis (Oppenheimer et al., 2007).

Neuroblastic tumors show dramatic neural maturation in response to RA through the transcriptional regulation of genes involved in the differentiation process (Oppenheimer et al., 2007; Reynolds et al., 2003). Many studies have concluded that the tyrosine kinase receptor RET plays a major role in differentiation and proliferation (Cerchia et al., 2006; Takahashi et al., 1991), and it is strongly activated by RA in neuroblastoma cells. Angrisano et al investigated the major epigenetic modifications occurring at RET locus in neuroblastoma cell line upon RA stimulation, and showed that a complex of series of molecular events, including modifications of both chromatin and DNA methylation state, accompany RA-mediated RET activation (Angrisano et al., 2011). Specifically, at RET promoter region, RA-induced RET activation included an increase in the methylation state of H3K4me3 (trimethylated lysine 4 on histone H3), while at RET enhancer region, a strong decrease of H3K27me3 marked RET gene activation upon RA exposure was observed. In addition, a cyclic increase of histone H3 acetylation state at both promoter and enhancer region was detected (Angrisano et al., 2011).

Another histone modification in response to all-trans RA (ATRA) was observed, this time in murine P19 embryonal carcinoma cells (Lefebvre et al., 2002). ATRA treatment did not significantly modulate the acetylation level of H3 and H4, but had a striking positive effect on phosphorylation of H3 at mRARβ2 promoter, leading to its transcriptional activation. In HeLa nuclear extracts, ARTA regulate protein acetylation events through inhibition of HDAC activity (Rahim and Strobl, 2009).

Reinforcement to the anticancer effect of RA through epigenetic mechanisms is indicated in "in vitro" studies conducted by Stefanska "et al", focusing on breast cancer cells (Stefanska et al., 2010; Stefanska et al., 2012). ATRA inhibited promoter methylation of RARβ2, a tumor suppressor gene which is frequently silenced due to promoter methylation during carcinogenesis, as well as increased the expression of RARβ2 (Stefanska et al., 2010). This effect of ATRA was relevant only in non-invasive MCF-7 cells (Stefanska et al., 2010). In the second study, the effect of ATRA on the methylation and expression of PTEN (phosphatase and tensin homologue) tumor suppressor gene was investigated (Stefanska et al., 2012). The authors found that in non-invasive MCF-7 cells, but not in highly invasive MDA-MB-231 cells, ATRA possesses high efficacy in the reduction of PTEN promoter methylation which might promote induction of their expression at early stages of carcinogenesis (Stefanska et al., 2012).

Similarly, Fazi et al showed that treatment with RA, which reprograms acute promyelocytic leukemia (APL) blasts to a nonleukemic phenotype, reduced DNMT expression and activity (Fazi et al., 2005). More specifically, RA treatment induced an early and coordinated decrease of DNMT1, DNMT3a and DNMT3b expression/activity, which correlated with demethylation of the promoter/exon-1 regions of its target gene RARβ2 in RA-responsive APL blasts cultured "in vitro", and in blood samples from one APL patient undergoing treatment with RA and chemotherapy. In the same manner, the following mechanism was suggested by Di Corce et al (Di Corce et al., 2002): oncogenic transcription factors aberrantly recruit DNMT's to target promoters. Newly methylated CpGs then become docking sites for methylbinding proteins, which in turn interact with both HDAC complexes and DNMT's. The assembled complexes could be further stabilized by interactions between DNMT's and HDAC1. If the initial recruitment step is not prevented, it may eventually lead to spreading of hypermethylation

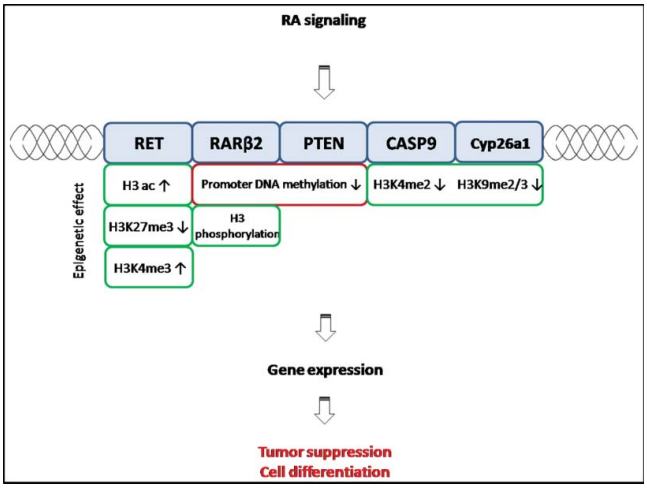

Figure 3. RA-mediated epigenetic changes in genes involving tumor suppression and cell differentiation. Relevant genes are presented in blue rectangles. Epigenetic effects in green and red represent histone modifications and DNA methylation, respectively.

to the neighboring regions, locking these into a stably silenced chromatin state.

In summary, binding of retinoids to RAR/RXR receptors initiates a cascade of changes in chromatin structure which can promote differentiation and initiate stable epigenetic changes (Zuchegna et al., 2014). In cancer cells, these changes have the potential to promote differentiation to a less neoplastically-transformed state (Figure 3). Retinoids act both by promoting stem cell differentiation and changing the pattern of gene expression in tumor cells to make them more sensitive to other therapies. As such, retinoids are likely to be a component in many future cancer therapies (Gudas and Wagner, 2011).

Vitamin A and immunity

There is now conclusive evidence that immunity is epigenetically regulated (Fernandez-Morera et al., 2010; Lal and Bromberg, 2009). These mechanisms are known to control both Th1 and Th2 cell differentiation, and are also prerequisite for FoxP3 (forkhead box P3) expression and Treg (regulatory T cell) differentiation.

Vitamin A and retinoids are recognized today as essential factors for normal immune system development and regulation (Iwata et al., 2004; Mora et al., 2003; Mora et al., 2006). For example, vitamin A is necessary for maintaining intestinal integrity (Reifen et al., 1998; Uni et al., 1998; Zaiger et al., 2004), regulating mucin gene expression (Amit-Romach et al., 2009; Gray et al., 2001), and ameliorating several models of autoimmunity, including inflammatory bowel disease, rheumatoid arthritis, and more (Racke et al., 1995; Zunino et al., 2007). RA is also involved in the regulation of T cell differentiation (Xiao et al., 2008); however, the molecular mechanisms by which RA exerts its effect are not fully understood. Vitamin A and RA generally promote differentiation toward Th2 cells and the production of IL-4 and IL-5 (Iwata et al., 2003; Ross, 2012; Stephensen et al., 2004) and also oppose Th17 cells commitment (Kimura and Kishimoto, 2010; Ross, 2012).

Within the past few years it has become evident that RA is one of the critical factors that provides signals for the

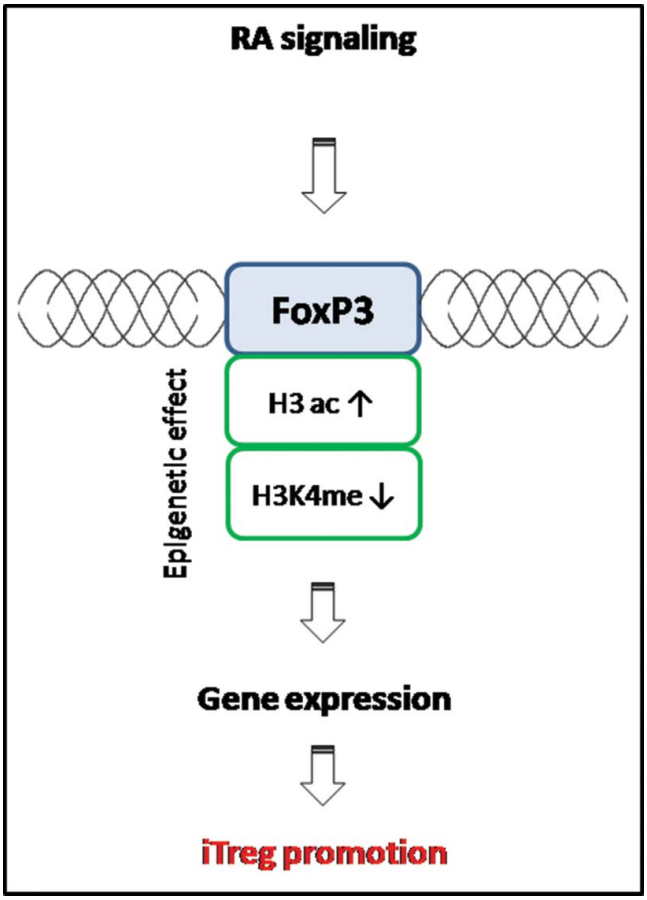

Figure 4. RA-mediated epigenetic changes in FoxP3 gene, involving immunity. Relevant genes are presented in blue rectangles. Epigenetic effects in green and red represent histone modifications and DNA methylation, respectively.

differentiation of iTreg cells (Figure 4). These cells are prominent at mucosal surfaces and typically perform a suppressive function; they are indispensable for self-tolerance and have been shown to suppress autoimmune disorders, and are marked by their expression of FoxP3 that is essential for their formation (Sakaguchi et al., 2010; Weaver and Hatton, 2009). Kang et al. showed that when FoxP3$^-$ T cells were cultured with ATRA, expression of FoxP3 mRNA was induced (Kang et al., 2007). This effect of ATRA was accompanied by a considerable enhancement of histone acetylation of the proximal and distal regions of the FoxP3 promoter, proving that vitamin A metabolites induce gut-homing FoxP3+ Treg cells at least partly by epigenetic mechanisms (Kang et al., 2007).3

Later on, Lu et al. showed that iTreg (inducible regulatory T cell) development and stability promoted by ATRA is not related to alteration of methylation status of CpG sites in the FoxP3 locus, but rather to posttranslational modification of histones (Lu et al., 2011). ATRA significantly increased methylation in histone H3K4 in the FoxP3 gene promoter, and upregulated the methylation of H3K4 at the FoxP3 locus. The authors also attributed the enhanced effect of ATRA on iTregs promotion to a significant increase observed in histone acetylation status of the FoxP3 promoter, as histone acetylation is considered an initial step in the relaxation of chromatin structure, often accompanied by gene transcription (Lu et al., 2011).

Table 3 Summary of epigenetic alterations mediated by retinoic acid.

	Epigenetic mechanism						
		Histone modification					
Model	DNA methylation	Methylation	Acetylation	Phosphorylation	Gene	Effect	Reference
hESCs	Average DNA methylation ↑ ↑				CHFR, Thy1	SC differentiation	(Cheon et al., 2010)
hESCs			H3K27 ↑		Hoxa1, Cyp26a1, RARβ2	SC differentiation	(Urvalec et al., 2010)
Mouse ESCs		H3K27me3 ↓ H3K4me3 ↑	H3, H4 ↑		Hoxa1	SC differentiation	(Lee et al., 2007)
F9 cells		H3K27me3 ↓			Hoxa1, Cyp26a1	RARβ2 induction	(Kashyap et al., 2010)
Neuroblastoma cells		H3K27me3 ↓ H3K4me3 ↑	H3 ↑		RET gene	RET activation Cell differentiation	(Angrisano et al., 2011)
P19 embryonic carcinoma cells				H3 ↑	mRARβ2	RARβ2 induction Tumor suppression	(Lefebvre et al., 2002)
MCF-7	Promoter methylation ↓				RARβ2	RARβ2 induction Tumor suppression	(Stefanska et al., 2010)
MCF-7	Promoter methylation ↓				PTEN	PTEN induction Tumor suppression	(Stefanska et al., 2012)
Acute promyelocytic leukemia cells	Promoter/exon-1 methylation ↓				RARβ2	RARβ2 induction Tumor suppression	(Fazi et al., 2005)
MCF-7		H3K4/9 demethylation-methylation cycles H3K4me2 H3K9me2/3 ↓			CASP9, Cyp2626A1	CASP9, Cyp2626A1 induction	(Zuchegna et al., 2014)
T cells			Promoter ↑		FoxP3	iTreg promotion	(Kang et al., 2007)
T cells		H3K4 at promoter & locus ↓	Promoter ↑		FoxP3	iTreg promotion	(Lu et al., 2011)

Together, these studies suggest that ATRA contributes to T cells differentiation through modulating epigenetic settings either in a locus-specific manner or globally on chromatin. Hence, the possible involvement of epigenetics in immune system regulation by vitamin A should be taken into consideration in future studies.

Summary

Epigenetics constitute the crossroads of cancer, inflammation and metabolic stress (Szarc vel Szic et al., 2010). It is thus likely that understanding and manipulating the epigenome has great potential in chemoprevention or stabilization of chronic inflammatory disorders. Such manipulation could be achieved through environmental conditions and diet that can affect transgenerational gene expression via "reversible" heritable epigenetic mechanisms.

The data presented here show that retinoic acid is a potent agent capable of inducing alterations in epigenetic modifications that produce various effects on the phenotype (Table 1, Figure 5). According to the current model of retinoid signaling (Dilworth and Chambon, 2001; Kashyap and Gudas, 2010; McKenna and O'Malley, 2002; Nagy and Schwabe, 2004), in the absence of RA, the RAR/RXR receptors interact directly with corresponsive elements [silencing mediator of retinoid and thyroid hormone receptors (SMRT), nuclear receptor corepressor (NCoR)] that recruit histone deacetylase complexes I/II, which deacetylate the lysine residues of histone tails. This enables a tight association with the nucleosome DNA and establishes a "closed" chromatin state that is inaccessible to transcription. The presence of RA releases these corepressors and induces histone acetylation, thus opening the compact chromatin structure that facilitates transcription (Kashyap and Gudas, 2010).

As we improve our understanding regarding the connections between diet and the epigenome, the opportunity for clinical applications will arise. Medical benefits of vitamin A as an epigenetic modulator, especially with respect to its chronic use as nutraceutical agent, should rely on our further understanding of its epigenetic effects during health and disease, as well as through different generations.

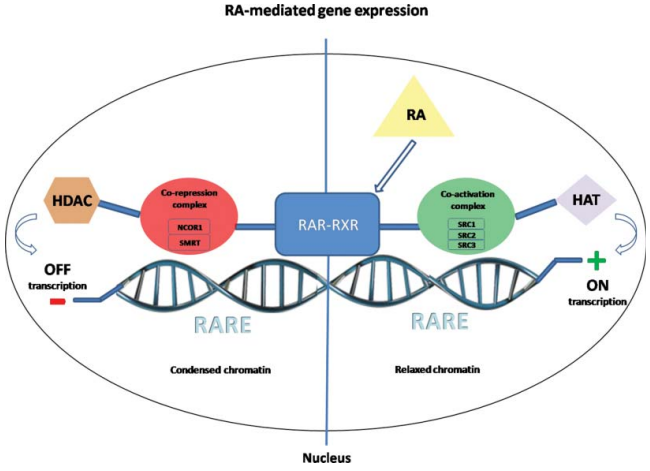

Figure 5. Summary of RA-mediated gene expression. SRC1/2/3- steroid receptor coactivator 1/2/3.

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