

Dietary vitamin A regulates wingless-related MMTV integration site signaling to alter the hair cycle

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Abstract

Alopecia areata (AA) is an autoimmune hair loss disease caused by a cell-mediated immune attack of the lower portion of the cycling hair follicle. Feeding mice 3–7 times the recommended level of dietary vitamin A accelerated the progression of AA in the graft-induced C3H/HeJ mouse model of AA. In this study, we also found that dietary vitamin A, in a dose dependent manner, activated the hair follicle stem cells (SCs) to induce the development and growth phase of the hair cycle (anagen), which may have made the hair follicle more susceptible to autoimmune attack. Our purpose here is to determine the mechanism by which dietary vitamin A regulates the hair cycle. We found that vitamin A in a dose-dependent manner increased nuclear localized beta-catenin (CTNNB1; a marker of canonical wingless-type Mouse Mammary Tumor Virus integration site family (WNT) signaling) and levels of WNT7A within the hair follicle bulge in these C3H/HeJ mice. These findings suggest that feeding mice high levels of dietary vitamin A increases WNT signaling to activate hair follicle SCs.

Keywords: Dietary vitamin A, hair cycling, hair follicle stem cell, WNT

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Introduction

The hair follicle requires vitamin A; as both vitamin A deficiency and excess lead to alopecia (hair loss).¹ The hair follicle cycles through three stages: growing phase (anagen), apoptosis phase (catagen), and resting phase (telogen).² Anagen can also be divided into three phases: development of the lower hair follicle (bulb; stages I–IIIa), development of the hair shaft (stages IIIb–V), and emergence of the hair shaft from the surface of the skin and its subsequent growth (stage VI).³ Alopecia occurs in all stages of the hair cycle, and different types of hair loss occur in different stages.

Cyclical alopecia during anagen develops with moderate increases in retinoids caused by blocking vitamin A storage in skin-specific acyl-CoA:diacylglycerol acyltransferase 1 (*Dgat1^{tm2^{Far}Tg}(KRT14-cre)1AMC*) null mice.⁴ In contrast, telogen effluvium occurs during oral treatment with at least either 25 mg/day of the synthetic retinoid acitretin (Soriatane) and its parent compound Etretinate,^{5,6} or 0.5 mg/kg body weight/day of isotretinoin.⁷ Telogen effluvium occurs due to an arrest of the hair follicle in telogen and an anchorage defect that allows the hair to fall out.⁵ Follicular hyperkeratosis develops during vitamin A deficiency in rats,^{8,9} juvenile cattle,¹⁰ and humans.^{11–13} Time course studies in rats suggest that the hair follicle is the last organ affected by vitamin A deficiency,¹⁴ which may

be why hair defects are rarely seen in vitamin A deficient humans. Combined, these studies suggest that different forms of alopecia occur when vitamin A is consumed at different doses.

Retinoic acid (RA) is the active metabolite of vitamin A, synthesized at or near its site of action, and can act in both autocrine and paracrine manners.¹⁵ RA acts primarily by binding its nuclear RA receptors (RARA, RARB, and RARG) and activating transcription.¹⁶ However, more recent studies suggest that RA can also activate kinase signaling pathways in the cytoplasm.¹⁷ One hair loss disease we previously examined is alopecia areata (AA). We found that feeding high (3–7 times recommended level) vitamin A accelerated disease; while feeding no vitamin A made the disease more severe.¹⁸ Similar to the eye, hair follicles are immune privileged and AA is argued to develop when that immune privilege collapses.¹⁹ AA develops when the anagen IIIa–VI hair follicle bulbs are attacked by cytotoxic CD8⁺NKG2D⁺ lymphocytes.²⁰ This destruction of the hair follicle leads to a breakage of the hair shaft resulting in exclamation mark hairs, cadaver hairs, and hair loss.^{21,22} Vitamin A plays many roles in the immune system,²³ which could be contributing to our previous results. In addition to these immune system effects, we found that feeding high vitamin A also altered the hair cycle.¹⁸ We found that high vitamin A increased the percentage of hair follicles in

anagen IIIa–VI. The anagen bulb is primarily attacked in AA. Therefore, high vitamin A may have accelerated the disease by increasing anagen induction or extending the length of anagen, and therefore the amount of time the hair follicle could be attacked. This finding needs to be further examined in patients with AA, as most humans with AA have fewer hair follicles in anagen.²⁴ We also reported that RA synthesis and signaling protein localization within the hair follicle was altered during the hair cycle,^{25,26} although few studies have examined how the hair cycle is regulated by vitamin A.

The hair cycle is maintained through the regulation of stem cells (SCs) in the hair follicle bulge.²⁷ Several factors regulate hair follicle SCs to ultimately regulate the hair cycle. Bone morphogenetic protein 2 (BMP2) from the subcutaneous fat, BMP4 from the dermis, BMP6 from the inner layer of the hair follicle, and several Wingless-related MMTV integration site (WNT) inhibitors within the SCs all act to keep hair follicle SCs in the quiescent state of telogen.^{28–30} Activation of these SCs occur when transforming growth factor beta 2 (TGFB2) and Noggin (NOG) inhibit BMP signaling and WNT ligands are secreted from the mesenchyme-derived dermal papilla, which sits below the SCs during telogen.^{31,32} WNT signaling then activates hair follicle SCs to induce the initial stages of anagen (anagen I).^{33,34} The canonical WNT signaling pathway involves the translocation of beta-catenin (CTNNB1) to the nucleus to activate transcription of target genes.³⁵ WNT7A and WNT7B are 2 of the 19 members of the WNT-secreted protein family that are important for initiation of anagen and are both directly inhibited by BMP signaling.^{36,37} RA regulates members of both the WNT and TGFB/BMP families in other tissues,^{38–42} but it is unknown which of these factors is regulated by RA in the hair follicle.

Our purpose here is to determine the mechanism by which dietary vitamin A regulates hair follicle SCs to induce anagen initiation. We are using control samples from our previous vitamin A feeding study in C3H/HeJ mice.¹⁸ We found that both nuclear localized CTNNB1 and WNT7A were dose dependently increased by dietary vitamin A. These findings extend the knowledge of vitamin A signaling in skin SCs.

Materials and methods

Mice

To induce AA, recipient C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, ME) received a dorsal skin graft from an older C3H/HeJ mouse that had spontaneously developed AA (sent from John P. Sundberg) as previously described.¹⁸ Control mice had their own dorsal skin removed and rotated 180° in a sham surgery. Recipient and control C3H/HeJ mice were fed an AIN93 Maintenance diet containing 0 ($n=8$), 4 ($n=7$), 12 ($n=9$), or 28 ($n=8$) IU vitamin A/g diet (Research Diets, Inc., New Brunswick, NJ) starting two weeks before these mice were grafted. The American Institute of Nutrition (AIN) recommends 4 IU vitamin A/g diet.⁴³ The higher doses were based on vitamin A analysis of the diet fed mice in The Jackson Laboratory production facility, which contained

6–28 IU vitamin A/g diet. Samples from the control sham mice collected 15 weeks post grafting were used in this current study. The Ohio State University Institutional Animal Care and Use Committee (IACUC) approved all procedures (#2008A0044).

Immunohistochemistry (IHC)

IHC was performed using a three-antibody system in paraffin slides as described previously.²⁵ The following antibodies were used: CTNNB1 (Millipore, Billerica, MA), WNT7A (Abcam, Cambridge, MA), WNT7B (Novus Biologicals, Littleton, CO), BMP4 (Vector Lab, Burlingame, CA), and TGFB2 (Santa Cruz, Biotechnology, Santa Cruz, CA). Biotinylated anti-rabbit and anti-mouse were used as secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) followed by a horseradish peroxidase-conjugated anti-biotin tertiary antibody (Bethyl Laboratories, Montgomery TX). The red 3-amino-9-ethylcarbazole plus enhancers (AEC+; Dako, Carpinteria, CA) substrate chromogen was used followed by counterstaining with Gills Hematoxylin III (Poly Scientific, Bay Shore, NY) and mounting in aquamount (Dako, Carpinteria, CA). CTNNB1 sections were not counterstained to better visualize nuclear localization. Immunoreactivity (IR) was scored blinded on a 4-point scale with: 0 = negative; 1 = weak; 2 = moderate; 3 = strong; and 4 = very strong. An IHC level (IHCL) was then calculated for nuclear CTNNB1 for each telogen hair follicle by the following formula

$$\begin{aligned} \text{IHCL} = & ((\# \text{ cells with IR of 0} / \text{total cells}) \times 0) \\ & + ((\# \text{ cells with IR of 1} / \text{total cells}) \times 1) \\ & + ((\# \text{ cells with IR of 2} / \text{total cells}) \times 2) \\ & + ((\# \text{ cells with IR of 3} / \text{total cells}) \times 3) \\ & + ((\# \text{ cells with IR of 4} / \text{total cells}) \times 4) \end{aligned}$$

The IHCL of all of the telogen hair follicles (19–64) were then averaged to get one IHCL per mouse. With this scoring method, the maximum IHCL is 4. The scoring system is modified from validated protocols.^{44–46} Blinded IHCLs for WNT7A, WNT7B, and TGFB were calculated similarly, but each hair follicle received one IR score and the formula summed the fraction of hair follicles with each IR score. A blinded IHC score of BMP4 was measured using the total number of BMP4 positive cells in the dermis around telogen hair follicles divided by the number of telogen hair follicles. Images were captured on an upright Olympus BX51 microscope with the DP71 camera (Olympus, Tokyo, Japan) and processed in Adobe Photoshop CS4 (San Francisco, CA).

Statistics

Comparison among experimental groups was analyzed by one-way analysis of variance, followed by Tukey's *post hoc* tests when appropriate using Statistical Package for the Social Sciences, v21 (IBM; Armonk, NY). *P* value < 0.05 is defined as statistical significance.

Results

To determine the mechanisms by which vitamin A regulates hair follicle SCs, we examined several factors known to regulate the hair cycle by IHC in mice fed various levels of vitamin A. Nuclear CTNNB1 is an important downstream component and marker of the active canonical WNT signaling pathway. We found that dietary vitamin A dose dependently increased the nuclear localization of CTNNB1 in C3H/HeJ mice with a significant increase occurring with as little as 4 IU vitamin A/g diet ($P < 0.001$) and an additional significant increase with 28 IU vitamin A/g diet (Figure 1(a–e)). WNT7A IR also increased with dietary vitamin A in a dose-dependent manner with C3H/HeJ mice fed 28 IU vitamin A/g diet being significantly greater than mice fed no vitamin A ($P < 0.05$, Figure 1(f–j)). WNT7B IR was not altered by the level of dietary vitamin A (Figure 1(k–o)). Dietary vitamin

A also did not significantly alter either BMP4 or TGFB2 (Figure 2).

Discussion

Vitamin A is important for the development and homeostasis of many tissues, including skin and hair.^{8,47,48} Our previous study found that dietary vitamin A-induced anagen in a dose-dependent manner in C3H/HeJ mice.¹⁸ Increased anagen was also seen in skin specific *Dgat1^{tm2^{Farrr}}Tg(KRT14-cre)IAMC* null mice, which have elevated retinol and RA levels.⁴ In our current study, we explored the mechanism by which dietary vitamin A regulates hair cycling. We found that dietary vitamin A increased WNT signaling, as indicated by nuclear CTNNB1, and the ligand WNT7A. Thus, high vitamin A increases the percentage of anagen hair follicles by increasing WNT signaling in the bulge SCs to induce the onset of anagen.

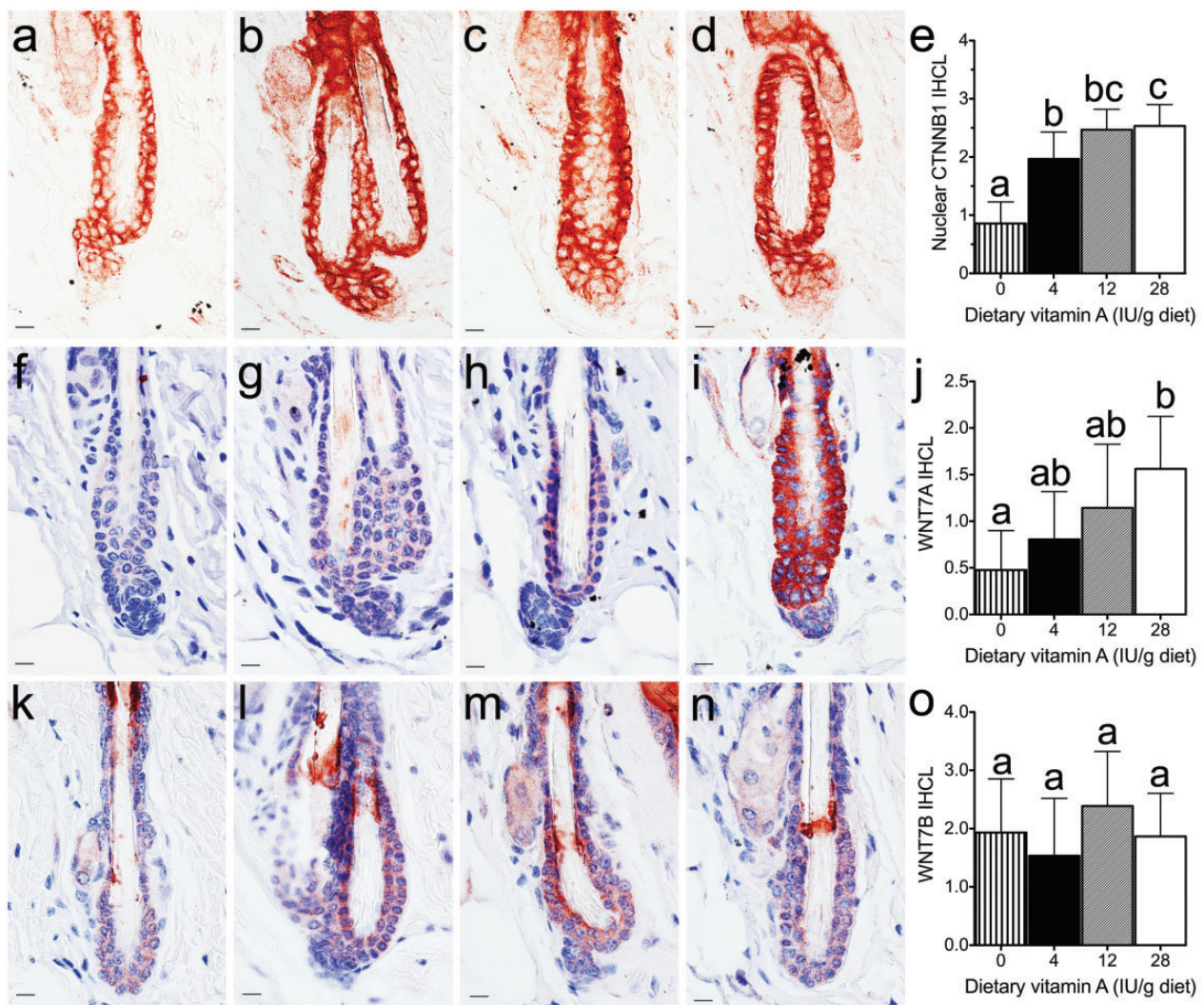


Figure 1 Dietary vitamin A dose dependently altered nuclear localized CTNNB1 and WNT7A in telogen hair follicles of C3H/HeJ mice. Immunohistochemistry was performed with antibodies against CTNNB1 (a–e), WNT7A (f–j), and WNT7B (k–o) in dorsal skin from C3H/HeJ mice fed 0 (a, f, k), 4 (b, g, l), 12 (c, h, m), and 28 (d, i, n) IU vitamin A/g diet. Blinded IHC score for CTNNB1 and WNT7A, WNT7B were calculated as the immunoreactivity (IR) measured on a 0–4 scale multiplied by the fraction of cells within the telogen hair follicle and the fraction of telogen hair follicles. Mean $\pm$ SD. Different letters are significantly different, $P < 0.05$. Bar = 10.1 μ m

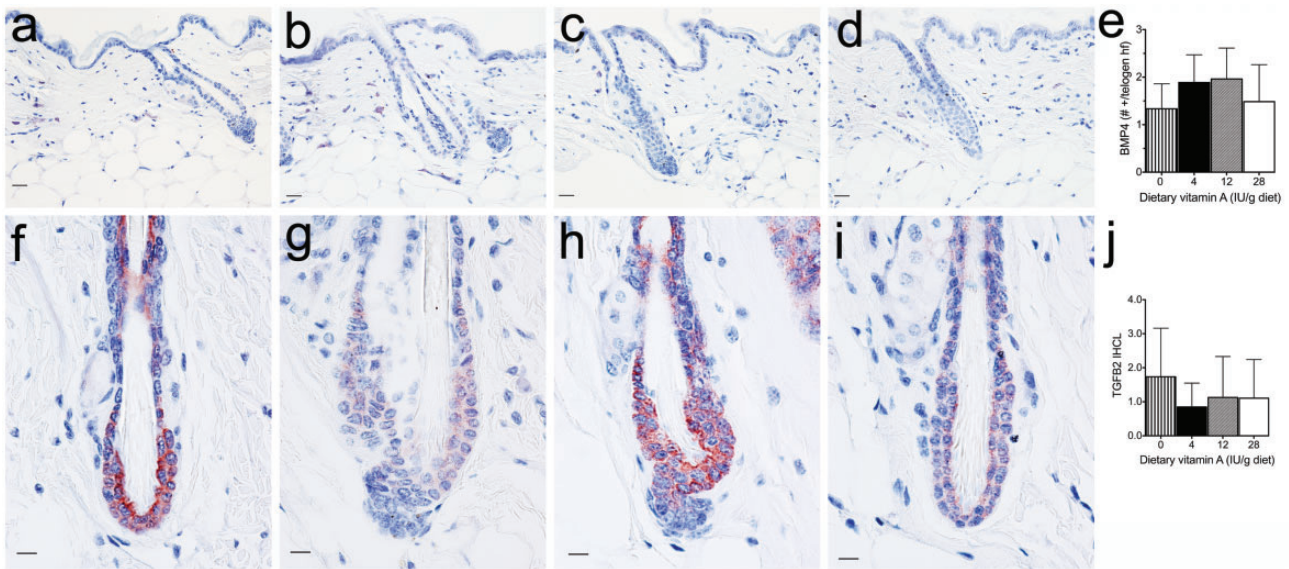


Figure 2 Dietary vitamin A did not significantly alter BMP4 or TGFB2 in telogen hair follicles of C3H/HeJ mice. Immunohistochemistry was performed with antibodies against BMP4 (a–e) and TGFB2 (f–j) in dorsal skin from C3H/HeJ mice fed 0 (a, f), 4 (b, g), 12 (c, h), and 28 (d, i) IU vitamin A/g diet. A blinded IHC score of BMP4 was measured using the total number of BMP4 positive cells in the dermis around telogen hair follicles divided by the number of telogen hair follicles. Blinded IHC score for TGFB2 was calculated as the immunoreactivity (IR) measured on a 0–4 scale multiplied by the fraction of cells within the telogen hair follicle and the fraction of telogen hair follicles. Mean $\pm$ SD. Bar = 10.1 μ M

We also observed that the alteration pattern of WNT7A expression by dietary vitamin A and that of CTNNB1 nuclear localization was similar in C3H/HeJ mice. However, the magnitude of change for CTNNB1 nuclear localization was greater. This suggests that other signals in addition to WNT7A may play a role in directing CTNNB1 to the nucleus. While WNT7A and WNT7B are essential to activate hair follicle bulge SCs,^{36,37} there are 19 members of the WNT secretion protein family that play different roles during hair follicle development and cycling.⁴⁹ Future studies could analyze different WNTs and WNT inhibitors.

The doses of vitamin A used in this study were 3–7 times the AIN recommendation.⁴³ In humans, consumption of vitamin A at 3–7 times the recommendation is at or above the established upper limit and potentially toxic.⁵⁰ But most mice consume these high levels on a regular basis because most standard unpurified diets contain extra vitamin A. Dr. Napoli calls the amount of vitamin A in unpurified diets “Copious”; and his group recently showed that these diets are causing lasting effects that impede vitamin A research.⁵¹ According to the National Health and Nutrition Examination Survey (NHANES), most Americans consume the recommended level of dietary vitamin A through diet alone. But a significant number of Americans receive 2–7 times the recommended level of vitamin A through supplements.⁵² These high levels of dietary vitamin A are on top of the retinoids that are delivered directly to the skin and hair via cosmetic products.

The interactions between RA and WNT signaling are tissue dependent.^{53,54} During skeletal myogenesis, osteogenesis, and adipogenesis, RA-activated WNT signaling.^{38,40} Yet during limb development, RA inhibited *Wnt7a*.⁴² Our study shows that RA is activating WNT7A

and WNT signaling within the adult skin *in vivo*. Several *Wnts* and WNT inhibitors were also increased in the skin of E16.5 and E18.5 *Cyp26b1* null embryos,⁵⁵ suggesting that RA regulates WNTs in the developing hair follicle as well. Future studies are needed to determine whether RA is acting directly on WNT7A within the hair follicle.

In summary, we demonstrated here that dietary vitamin A activates hair follicle SCs dose dependently through the activation of WNT signaling. Our next step is to study the mechanism by which retinoids regulate WNT. We will also establish the healthy range of retinoids for the hair follicle, which could provide possible knowledge for maintaining healthy hair in the normal population and determine effective doses of retinoids in treating other hair loss diseases.

Author contributions: All authors participated in the design, interpretation of the studies, and analysis of the data and review of the manuscript; LS and HBE conducted research; LS and HBE analyzed data; LS and HBE wrote the manuscript; JPS provided C3H/HeJ mice for the surgeries and edited the manuscript; HBE had primary responsibility for final content. All authors read and approved the final manuscript.

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