9-*cis* Retinoic Acid is the ALDH1A1 Product that Stimulates Melanogenesis

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Abstract

Aldehyde dehydrogenase 1A1 (ALDH1A1), an enzyme that catalyzes the conversion of lipid aldehydes to lipid carboxylic acids, plays pleiotropic roles in UV-radiation resistance, melanogenesis, and stem cell maintenance. In this study, a combination of RNAi and pharmacologic approaches were used to determine which ALDH1A1 substrates and products regulate melanogenesis. Initial studies revealed that neither the UV-induced lipid aldehyde 4-hydroxy-2-nonenal nor the ALDH1A1 product all-*trans* retinoic acid appreciably induced melanogenesis. In contrast, both the ALDH1A1 substrate 9-*cis* retinal and its corresponding product 9-*cis* retinoic acid potently induced the accumulation of *MITF* mRNA, *Tyrosinase* mRNA, and melanin. ALDH1A1 depletion inhibited the ability of 9-*cis* retinal but not 9-*cis* retinoic acid to stimulate melanogenesis, indicating that ALDH1A1 regulates melanogenesis by catalyzing the conversion of 9-*cis* retinal to 9-*cis* retinoic acid. The addition of potent ALDH1A inhibitors (cyanamide or Angeli's salt) suppressed *Tyrosinase* and *MITF* mRNA accumulation *in vitro* and also melanin accumulation in skin equivalents, suggesting that 9-*cis* retinoids regulate melanogenesis in the intact epidermis. Taken together, these studies not only identify cyanamide as a potential novel treatment for hyperpigmentary disorders, but also identify 9-*cis* retinoic acid as a pigment stimulatory agent that may have clinical utility in the treatment of hypopigmentary disorders, such as vitiligo.

Keywords

Introduction

Melanin, a pigment produced by the epidermal melanocyte, protects the skin from the harmful effects of UV irradiation and is aberrantly regulated in many human skin diseases including vitiligo and melasma (1, 2). Genetic and biochemical studies have identified over 150 genes that regulate melanogenesis in mouse and human skin (3). This knowledge has led to the identification of pigment inhibitory agents, such as hydroquinone, that can effectively inhibit melanin production *in vivo* (3). Unfortunately, the clinical use of hydroquinone has been limited by its potential teratogenicity (4–7). Despite the fact that many genes that regulate melanogenesis have been identified, effective pharmacologic agents that can stimulate melanogenesis in hypopigmented skin are currently unavailable (8,

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9). These observations highlight the continuing need to develop safe pigment modulatory agents for the treatment of pigmentary disorders.

Our group recently utilized a genome-wide RNAi screening approach to identify pharmaceutically tractable drug targets for the rational design of pigment modulatory agents (10). This approach identified 92 novel regulators of melanin production in human cells, including a number of genes that control the expression of microphthalmia-associated transcription factor (MITF), the central transcriptional regulator of melanogenesis, and tyrosinase (TYR), the enzyme that catalyzes the rate-limiting step in melanogenesis (1, 10–12). One of the novel regulators of MITF and TYR expression identified in our screen was ALDH1A1 (10), a well-characterized NAD $(P)^+$-dependent enzyme that catalyzes the irreversible oxidation of highly reactive aliphatic and aromatic aldehydes to their corresponding non-toxic carboxylic acids (EC number 1.2.1.36) (13). The ALDH1A subfamily (ALDH1A1, ALDH1A2, ALDH1A3) is known to catalyze, with an equal efficiency, the oxidation of all-*trans* retinal and 9-*cis* retinal to all-*trans* retinoic acid and 9-*cis* retinoic acid, respectively (14, 15). Additionally, ALDH1A1 functions in the lens and cornea to detoxify 4-hydroxy-2-nonenal (4-HNE), a highly reactive lipid aldehyde that is generated from ultraviolet radiation induced lipid peroxidation reactions (16, 17). Other studies have revealed that ALDH1A1 is a marker of stem cells (18). In summary, ALDH1A1 is an enzyme that is able to regulate diverse processes ranging from stem cell maintenance (18), to UV-radiation resistance, to melanogenesis (10) by catalyzing the conversion of specific aldehydes to their corresponding carboxylic acids (1, 10, 16–20).

Previous work demonstrated that ALDH1A1 depletion in primary melanocytes and MNT-1 melanoma cells inhibited the accumulation of both *TYR* and *MITF* mRNA and protein (10). Additionally, two potent inhibitors of the ALDH1A family (cyanamide and Angeli's salt) were shown to inhibit both TYR protein accumulation and melanin accumulation in normal human melanocytes (10). Recent studies have suggested that human melanocytes can catalyze the light-induced isomerization of 9-*cis* retinal and revealed that the addition of 9-*cis* retinal in conjunction with UVA radiation is sufficient to induce melanin accumulation in normal human melanocytes (21). In this study, we utilize a combination of RNAi and pharmacologic approaches to identify 9-*cis* retinal as the ALDH1A1 substrate that regulates melanogenesis.

Materials and Methods

Cell culture

Human MNT-1 melanoma cells were a gift from M. Marks (University of Pennsylvania). These cells were cultured in DMEM (CellGro) supplemented with 15% fetal bovine serum (CellGro), sodium pyruvate (Invitrogen), L-glutamine (Invitrogen), MEM vitamin solution (Invitrogen), antibiotic-antimycotic (Invitrogen), and 10% AIM-V medium (Invitrogen). For the melanin quantitation experiments, MNT-1 cells were switched to DMEM minus phenol red (CellGro) supplemented with 10% fetal bovine serum, sodium pyruvate, L-glutamine and antibiotic-antimycotic as previously described (10). Human deeply-pigmented neonatal epidermal melanocytes (Invitrogen) were cultured in Medium 254 (Invitrogen) supplemented with phorbol 12-myristate 13-acetate-free Human Melanocyte growth supplement-2 (Invitrogen). As these melanocyte strains were purchased from commercial entities, no IRB approval was required prior to their use.

Drug Treatment

Briefly, MNT-1 cells or deeply-pigmented (DP) melanocytes were plated at a density of 1.5 $\times 10^4$ cells per well of a 96-well microtiter plate and allowed to re-attach overnight.

Subsequently, cells were incubated with varying concentrations of cyanamide (Sigma Aldrich), Angeli's salt (Sodium α-oxyhyponitrite, chemical name; disodium diazen-1-ium-1,2,2-triolate, formal name; Cayman Chemical), 4-HNE (4-hydroxy-2E-nonenal, formal name; Cayman Chemical), 9-*cis* retinal, 9-*cis* retinoic acid, all-*trans* retinal or all-*trans* retinoic acid at the indicated concentrations (Sigma Aldrich). The cells were incubated with the retinoids for merely 30–45 minutes to avoid cellular toxicity. In the case of cyanamide, Angeli's salt and 4-HNE, cells were incubated in each drug for at least 24 hours. For the UV experiments, cells were incubated in media containing 9-*cis* retinal for 30 minutes before being irradiated with the indicated dose of UV light. Media was immediately refreshed after UV irradiation, and RNA was isolated from these cells 24 hours later. For the melanin quantitation experiments, cells were allowed to grow to confluency post 9-*cis* retinal/UVA treatment.

Real-time quantitative PCR for mRNA

A Cells-to-Ct kit was utilized to lyse the cells after the appropriate drug treatment (Applied Biosystems). A high capacity RNA-to-cDNA kit was then employed to generate cDNA (Applied Biosystems). Solaris qPCR gene expression assays for MITF (AX-008674-00-0100), TYR (AX-012555-00-0200), ALDH1A1 (AX-008722-00-0100), β-actin (AX-003451-00-0100) and GAPDH (AX-004253-00-0100) were obtained from Thermo Scientific (Dharmacon RNAi technologies) and used with TaqMan Gene Expression Master Mix (Applied Biosystems) to complete the PCR reaction. A 7900HT Fast Real-Time PCR system (Applied Biosystems) and SDS 2.4 (Applied Biosystems) were used to determine Ct values for each sample. Values were normalized to either β-actin or GAPDH using the relative quantification mathematical model (Pfaffl) as previously described (10). A two-tailed Student's *t*-test was employed to determine statistical significance.

Western blotting

MNT-1 or DP melanocytes were plated in 96-well plates at a concentration of 2×10^4 cells per well, allowed to re-attach overnight, and then incubated with drug for 30 hours. After treatment, the number of cells in each well was quantified and lysates were prepared using the RIPA lysis buffer system (RIPA supplemented with protease inhibitor, PMSF and sodium orthovanadate; Santa Cruz Biotechnology). Lysates were then clarified by centrifugation (3,000 rpm for 10 minutes at 4°C). The relative concentration of protein in each lysate was quantified using both a BCA Protein Assay Kit (Thermo Scientific) and a Coomassie (Bradford) protein assay (Thermo Scientific) to eliminate the effects of interfering melanin. A total of 20 μg of protein per sample was separated on a 4–20% SDS-polyacrylamide gel (Bio-Rad) under reducing conditions and transferred onto to a 0.45-μm nitrocellulose membrane (Bio-Rad). Subsequently, membranes were blocked in a blocking buffer solution comprised of TBS (Fisher Scientific), 0.1% Tween-20 (Fisher Scientific) and 5% non-fat milk powder (Apex).

The following antibodies were used: rabbit polyclonal α/β-tubulin, #2148, Cell Signaling Technology; goat anti-rabbit IgG, HRP-linked, #7074, Cell Signaling Technology; mouse monoclonal tyrosinase, sc-20035, Santa Cruz Biotechnology; bovine anti-mouse IgG-HRP sc-2371, Santa Cruz Biotechnology. To assess immunoreactivity, either a SuperSignal West Dura Extended Duration Substrate or a SuperSignal West Pico Chemiluminescent Substrate was used according to manufacturer's directions (Pierce Protein Biology Products). Protein levels were assessed using densitometry analysis (ImageJ).

RNA interference

1.5×10^4 MNT-1 melanoma cells were reverse-transfected in 96-well microtiter plates with 50 nM ALDH1A1 or control siRNAs using a Dharmafect 2 transfection reagent as previously described (10). A non-targeting control (SMARTpool; D-001810-10-20; Thermo Scientific, Dharmacon RNAi Technologies) was used as the negative control, while three different siRNA oligos purchased from Ambion (s1236, s1237, s1238) were pooled for the ALDH1A1 depletion experiments. Transfected cells were allowed to remain in the transfection media for 72 hours before drug treatment to ensure protein knockdown. Real-time quantitative RT-PCR was used to verify that ALDH1A1 siRNA effectively inhibited the expression of ALDH1A1.

Pigment Measurement

MNT-1 cells were plated at a density of 1×10^4 cells per well in a 96-well plate, allowed to re-attach overnight, and incubated in the indicated concentration of drug for four days. Cells were then lysed using a Cell-Titer Glo reagent (Promega), and relative melanin accumulation was quantified by measuring the absorbance of each well at 405 nm and normalizing this value to the relative cell number in each well as determined by the Cell-Titer Glo assay (10). For the UVA experiments, cells were incubated with the indicated concentration of 9-*cis* retinal for 30 minutes, and then irradiated with the indicated dose of UVA. Media was changed immediately after UVA treatment to avoid toxicity. Cells were then lysed four days after UVA treatment according to the protocol described above. The relative pigment index (PI) was calculated by dividing the absorbance values at 405 nm by the Cell-Titer Glo luminescence values, and then normalizing these values to the experimental control (vehicle-treated wells). To calculate the % pigment induction, the following equation was used: (PI of test – PI control (vehicle)) x 100%. A Student's two-tailed *t*-test was used to calculate the statistical significance of each value compared to the vehicle-treated control.

Maintenance and treatment of three-dimensional skin equivalents

MelanoDerm deeply-pigmented three-dimensional skin equivalents were obtained from MatTek Corporation (Ashland, MA) and maintained according to the manufacturer's instructions. The equivalents were maintained in a humidified incubator at 37°C with 5% CO_2 in EPI-100-NMM-113 media as prepared by MatTek (DMEM with 5 µg/mL gentamicin, 0.25 µg/mL amphotericin, α-MSH, β-FGF, and KGF). Both the media and the drug were refreshed every other day for three weeks before the samples were harvested for a solvable melanin assay. Cyanamide (600 µM) and kojic acid (2%) were dissolved in water before being added to the equivalents at a total volume of 20 µL. The relative melanin content of each skin equivalent after drug treatment was determined using a solvable melanin assay (22). A Student's two-tailed *t*-test was utilized to determine whether cyanamide or kojic acid significantly inhibited the accumulation of melanin in skin equivalents.

Results

9-*cis* retinal is the ALDH1A1 substrate that stimulates melanogenesis

While published studies have defined lipid aldehydes that are detoxified by ALDH1A1 and identified ALDH1A1 substrates that promote cellular differentiation (17, 19, 23, 24), it is currently unclear which ALDH1A1 substrates regulate melanogenesis. Initial studies sought to verify that the ALDH1A family regulates melanogenesis by a catalytic mechanism. Two potent inhibitors of ALDH1A, cyanamide and Angeli's salt, inhibited the accumulation of *TYR* and *MITF* mRNA in a dose responsive manner (Figures 1A–1B), consistent with

previous observations that ALDH1A1 depletion inhibited the accumulation of TYR and MITF protein (10).

Once we had determined that ALDH1A inhibitors could block melanogenesis, we next sought to identify putative substrates of ALDH1A that regulate melanogenesis. ALDH1A1 exhibits a high affinity for 4-HNE, one of the most abundant α, β-unsaturated aldehydes generated from UV-induced lipid peroxidation reactions (17, 19, 20). Interestingly, 4-HNE not only inhibited the accumulation of melanin (Figure S1A) but also the accumulation of *TYR* and *MITF* mRNA in MNT-1 cells (Figure 1C), indicating that ALDH1A regulates melanogenesis by a mechanism that is distinct from its role in inhibiting corneal opacification (16, 17, 19).

We next sought to determine whether other known ALDH1A substrates stimulate melanogenesis. In addition to 4-HNE, ALDH1A also catalyzes the oxidation of retinal to retinoic acid (15, 25, 26). Notably, ALDH1A can metabolize both all-*trans* retinal and 9-*cis* retinal to their corresponding carboxylic acids with equal efficiency (14). Recently published studies have demonstrated that 9-*cis* retinal can promote melanin accumulation in the context of UVA irradiation (14, 21). Therefore, we sought to define specific retinaldehydes that stimulate melanogenesis. All-*trans* retinal stimulated melanogenesis at relatively high doses (Figure S1B). These observations could be secondary to a direct stimulatory effect of all-*trans* retinal on melanogenesis, or secondary to the fact that all-*trans* retinal can be isomerized to 9-*cis* retinal, as suggested by others (21, 27). Interestingly, 9-*cis* retinal in conjunction with UVA irradiation stimulated the accumulation of *TYR* and *MITF* mRNA in both MNT-1 melanoma cells and primary melanocytes (Figures 1D, S1C). Similarly, the combination of 9-*cis* retinal treatment and UVA exposure induced melanin accumulation in MNT-1 cells, even though this treatment also induced cellular cytotoxicity (Figures 1E, S2A, S2B).

The metabolic product of 9-*cis* retinal oxidation, 9-*cis* retinoic acid, acts to stimulate melanin accumulation

While *cis*-retinal isomerization to *trans*-retinal is UV dependent, the oxidation of retinal by ALDH1A is not (28). In order to better establish whether 9-*cis* retinal or all-*trans* retinal regulates melanogenesis, we next sought to determine whether 9-*cis* retinal could stimulate melanin accumulation in the absence of UV-induced isomerization. 9-*cis* retinal induced pigment accumulation in a dose-responsive manner in the absence of UV irradiation (Figure 2A). As 9-*cis* retinal was able to induce pigment accumulation independently of UV-irradiation, we reasoned that the addition of 9-*cis* retinoic acid, the oxidation product of 9-*cis* retinal catalyzed by ALDH1A (15, 25), should also stimulate melanin accumulation. As expected, 9-*cis* retinoic acid stimulated melanin accumulation in MNT-1 cells both visually and quantitatively (Figures 2B, 2C). Interestingly, 9-*cis* retinoic acid stimulated melanin accumulation more effectively than 9-*cis* retinal, providing evidence that the product of ALDH1A catalysis is the actual species that stimulates melanogenesis (Figures 2A, 2B). In contrast, all-*trans* retinoic acid did not induce melanin accumulation (Figure 2D), providing further evidence that 9-*cis* retinoids and not all-*trans* retinoids stimulate melanogenesis.

9-*cis* retinoic acid stimulates the accumulation of *TYR* and *MITF* mRNA and TYR protein

Based on the observation that both 9-*cis* retinal and 9-*cis* retinoic acid stimulated melanin accumulation in the absence of UVA radiation, we reasoned that the 9-*cis* retinoids should also induce the accumulation of *TYR* and *MITF* mRNA. As predicted, both 9-*cis* retinal and 9-*cis* retinoic acid induced the accumulation of *TYR* (Figures 3A, 3C) and *MITF* (Figures 3B, 3D, 3E) mRNAs in both primary melanocytes and MNT-1 melanoma cells, demonstrating that either species alone is sufficient to stimulate the transcription of critical

regulators of melanogenesis. 9-*cis* retinal was not sufficient to induce TYR protein accumulation in either MNT-1 cells (upper panel) or primary melanocytes (lower panel) (Figure 3F). However, 9-*cis* retinoic acid induced the accumulation of TYR protein in a dose responsive manner in both skin cell types (Figure 3F). These observations could either be secondary to the fact that only a small fraction of 9-*cis* retinal is converted to 9-*cis* retinoic acid or due to the fact that the slow kinetics of the reaction prevented us from observing the stimulatory effect of 9-*cis* retinal on TYR protein accumulation within the experimental timeframe. Nonetheless, these results suggest that ALDH1A1 regulates melanogenesis by catalyzing the conversion of 9-*cis* retinal to 9-*cis* retinoic acid.

9-*cis* retinoic acid is able to stimulate the accumulation of *TYR* and *MITF* mRNAs in the absence of endogenous levels of ALDH1A1

To verify that ALDH1A regulates melanogenesis via the oxidation of 9-*cis* retinal, we examined whether 9-*cis* retinal could induce melanogenesis in an ALDH1A1 independent manner. Initial studies identified pooled ALDH1A1 siRNAs that effectively inhibited the expression of ALDH1A1 mRNA (Fig S2C). As theorized, ALDH1A1 depletion inhibited the ability of 9-*cis* retinal (Figure 3G) but not of 9-*cis* retinoic acid (Figure 3H) to stimulate the accumulation of *TYR* and *MITF* mRNA. These results confirm that the product of ALDH1A1 oxidation, 9-*cis* retinoic acid, stimulates melanogenesis, because the addition of 9-*cis* retinal in the absence of ALDH1A1 did not induce the accumulation of *TYR* and *MITF* mRNAs.

Cyanamide is able to inhibit the production of melanin in a three-dimensional model of human skin

Once we had determined how ALDH1A regulates melanogenesis *in vitro*, we next sought to determine whether ALDH1A activity regulates melanogenesis in a more physiologic model of human skin. For these experiments, we obtained skin equivalents containing normal human epidermal keratinocytes and melanocytes from Mattek, an equivalent model that has been used by other groups to assess the efficacy of skin depigmenting agents (22). Using doses that had been used previously to inhibit melanogenesis in primary melanocytes (10), we determined that cyanamide was able to inhibit melanin accumulation quantitatively as measured by a solvable melanin assay (Fig 4A), as well as both visually and histologically as measured by Fontana Masson staining (Figure 4B).

Additionally, we sought to investigate whether 9-*cis* retinoic acid could induce melanogenesis in human skin equivalents. Preliminary studies revealed that neither forskolin (a well established pigment agonist) (29) nor 9-*cis* retinoic acid is able to quantitatively stimulate melanogenesis in lightly or darkly pigmented skin equivalents (data not shown). While an abundance of literature has documented the utility of skin equivalent models for quantifying the efficacy of melanogenesis inhibitors (22, 30–34), this system has not yet been optimized to quantify the efficacy of pigment agonists. Our preliminary results suggest that further optimization of these models is required before they can be used to quantify the efficacy of pharmacologic agents that induce melanogenesis.

Discussion

In this study, we sought to define ALDH1A substrates and products that regulate melanogenesis. Initial studies revealed that 9-*cis* retinal and 9-*cis* retinoic acid stimulated melanin accumulation, while all-*trans* retinoic acid was unable to stimulate melanin accumulation (Figures 1E, S2A, 2A, 2B, 2D). These results identify a novel role for the 9-*cis* retinoids in regulating melanogenesis. Consistent with this observation, we observed that

the addition of 9-*cis* retinoic acid potently induced the accumulation of *MITF* and *TYR* mRNA, and TYR protein in melanocytic cells (Figures 3C–3F).

9-*cis* retinal was able to induce the accumulation of *MITF* and *TYR* mRNA at high doses, but was unable to induce the accumulation of TYR protein (Figures 3A, 3B, 3F). It is currently unclear what percent of the added 9-*cis* retinal is converted to 9-*cis* retinoic acid in our experiments. This makes it difficult to compare the relative effects of 9-*cis* retinal and 9-*cis* retinoic acid on the accumulation of *MITF* and *TYR* mRNA. Moreover, the interpretation of the results of these experiments is further complicated by the fact that the added 9-*cis* retinoic acid could also be converted to all-*trans* retinoic acid (35), and all-*trans* retinoic acid and 9-*cis* retinoic acid can synergistically activate RXR-RAR heterodimers (35, 36).

In light of these complications, we sought to better clarify whether 9-*cis* retinoic acid independently regulates melanogenesis. Depletion of ALDH1A1 inhibited the ability of 9-*cis* retinal but not 9-*cis* retinoic acid to stimulate melanogenesis (Figures 3G, 3H). This indicates that 9-*cis* retinoic specifically stimulates melanogenesis. Finally, ALDH1A inhibitors also inhibited the accumulation of *MITF* and *TYR* mRNAs *in vitro* and melanin accumulation in skin equivalents, further implicating a role for 9-*cis* retinoic acid in stimulating melanogenesis (Figures 1A–1B, 4A–B). Future studies will establish better models to examine the independent effects of different retinoid isomers on melanogenesis in human skin.

A multitude of studies to date have sought to determine whether all-*trans* retinoic acid inhibits or stimulates melanogenesis. While a number of studies demonstrate that all-*trans* retinoic acid inhibits melanogenesis (37–40), an equal number of studies propose that it stimulates melanogenesis or augments UV-induced melanogenesis in a variety of mouse and human cell lines (41–44). In this study, we approached this question by attempting to identify ALDH1A1 substrates and products that regulate melanogenesis. Our results revealed that all-*trans* retinoic acid was able to stimulate melanogenesis at high doses, while 9-*cis* retinoic acid was able to potently stimulate melanogenesis. It is particularly intriguing that 9-*cis* retinoic acid, which is not thought to have a functional role in the physiology of epidermal keratinocytes (45), appears to have a specific role in regulating the differentiation of epidermal melanocytes. Future studies will determine whether 9-*cis* retinoic acid related agents (rexinoids) are selective agents that can induce melanogenesis.

Extensive studies have sought to better define how retinoids and rexinoids regulate transcription. The biological effects of 9-*cis* retinoic acid are primarily mediated by its cognate receptor, the retinoid X receptor (RXR), of which there are three isoforms (α, β, and γ) (23, 46). While the retinoic acid receptor (RAR) can be activated by either 9-*cis* retinoic acid or all-*trans* retinoic acid, RXRs are activated exclusively by 9-*cis* retinoic acid (46, 47). Along with RARs, RXRs are members of the steroid hormone receptor superfamily of ligand-activated transcription factors (24, 48). RXRs are known to dimerize with a variety of nuclear receptors, including the vitamin D receptor (VDR), the peroxisome proliferator-activated receptor (PPAR), and the thyroid hormone receptor (T3R) (49–51). RXR homodimers act as potent inhibitors of transcription in the absence of ligand and only activate transcription upon ligand binding (52).

In the absence of ligand, RXR interacts with co-repressor proteins including silencing mediator for retinoid and thyroid hormone receptors (SMRT) and nuclear receptor co-repressor (N-CoR) (52, 53). These homologous proteins mediate the repressive effect of un-liganded receptors by recruiting histone deacetylase complexes to effectively silence the chromatin (52, 53). Upon ligand binding, RXR recruits such proteins as CBP/p300, p300/

CBP-associated factor and members of the SRC/p160 family (SRC-1/NCoA-1, TIF-2/GRIP-1/NCoA-2/SRC-2 and pCIP/ACTR/A1B1/TRAM1/RAC3/SRC-3), which possess intrinsic histone acetylase transferase activity and potentiate the transcriptional activity of ligand-bound receptors (52, 54, 55).

Interestingly, when we searched the MITF promoter region for putative RXR binding sites, we discovered that while this promoter did not contain RXR-VDR or RXR-T3R binding sites, it did contain a highly conserved RAR-RXR heterodimer binding site located proximal to the transcription start site (56, 57). While only RAR agonists are required to activate transcription downstream of RAR-RXR heterodimers, it has been demonstrated that the addition of RAR and RXR agonists can synergistically stimulate transcription from these sites (58, 59). Other studies have determined that the vitamin D receptor (VDR) binds its enhancer element as a heterodimer with RXR in the human epidermis, while the thyroid hormone receptor, another potential binding partner of RXR, is expressed in the epidermis (60–62). Taken together, these studies indicate not only that RXR can play pleiotropic roles in regulating the differentiation of the epidermis but also that agents that can stimulate both RAR and RXR may be more effective at inducing melanogenesis as compared to RAR agonists alone.

Although there are three unique isoforms of RXR (α, β, and γ) RXRα is the most abundant isoform expressed in the epidermis (24, 50, 51). Interestingly, RXRα mutant mice demonstrate a premature greying phenotype, which is first visible in the snout hairs and then spreads to the truncal hair (63). Subsequent to the hair graying, these mice develop a rapidly progressive alopecia characterized by the widespread accumulation of keratinous cysts (63). Keratinocyte lineage-specific RXRα knockout mice develop progressive alopecia and accumulate keratinous cysts but do not exhibit premature hair graying (64, 65), suggesting that RXRα may have independent effects on melanogenesis and keratinocyte differentiation.

Vitiligo is characterized by the destruction of melanocytes (66). New melanocytes must migrate into the de-pigmented area of skin before re-pigmentation can occur (66). The major reservoir for melanocyte replacement is within the outer root sheath or bulge area of the hair follicle (66). Previous studies have demonstrated that retinoic acid stimulates the differentiation of melanoblasts to melanocytes as characterized by the synthesis of pigment in the melanoblasts (67). When coupled with our observations that RXR agonists can specifically stimulate melanogenesis in melanocytes, our studies implicate a role for RXR agonists as drugs with the potential to re-induce melanogenesis in vitiliginous skin. Future studies will optimize existing skin equivalent models to quantitatively determine whether RXR agonists can stimulate melanogenesis in skin equivalents with the goal of developing selective agents that can induce melanogenesis in vitiliginous skin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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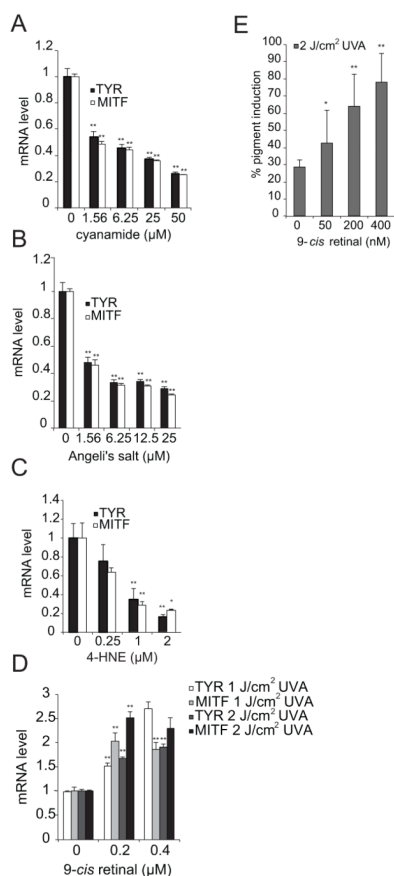
Figure 1. 9-*cis* retinal is the ALDH1A1 substrate that stimulates melanogenesis
(A) The ALDH1A inhibitor cyanamide inhibits *TYR* and *MITF* mRNA accumulation.
MNT-1 melanoma cells were treated with the indicated doses of cyanamide for 24 hours and
the relative expression of *TYR* and *MITF* mRNA was quantified as described in the
methods. **(B) The ALDH1A inhibitor Angeli's salt inhibits *TYR* and *MITF* mRNA
accumulation.** MNT-1 melanoma cells were treated with the indicated doses of Angeli's
salt for 24 hours and the relative expression of *TYR* and *MITF* mRNA was quantified as
described in the methods. **(C) 4-HNE is not sufficient to induce the accumulation of
either *TYR* or *MITF* mRNA**. MNT-1 melanoma cells were treated with the indicated doses
of 4-HNE for 24 hours, and *TYR* and *MITF* mRNA expression was quantified as described
in the methods. **(D) In conjunction with UVA radiation, 9-*cis* retinal induces the
accumulation of both *TYR* and *MITF* mRNA.** MNT-1 melanoma cells were pre-incubated
with 9-*cis* retinal at the indicated concentrations for 45 minutes before being irradiated with
UVA light as indicated. Relative mRNA expression was quantified as described in the
methods. All data shown are mean $\pm$ S.D. (n=3 as indicated by the *error bars*). *, $p < 0.05$ or
**, $p < 0.01$ using a Student's paired t-test with a two-tailed normal distribution *versus*
vehicle-treated control. **(E) Melanin accumulation is induced by 9-*cis* retinal in
conjunction with UVA radiation.** MNT-1 melanoma cells were treated with the indicated
doses of 9-*cis* retinal, irradiated with UVA, and then allowed to reach confluency. Melanin
accumulation was measured as described in the methods section. Data shown are mean $\pm$
S.D. (*n*=6 as indicated by the *error bars*). *, $p < 0.05$ or **, $p < 0.01$ using a Student's paired
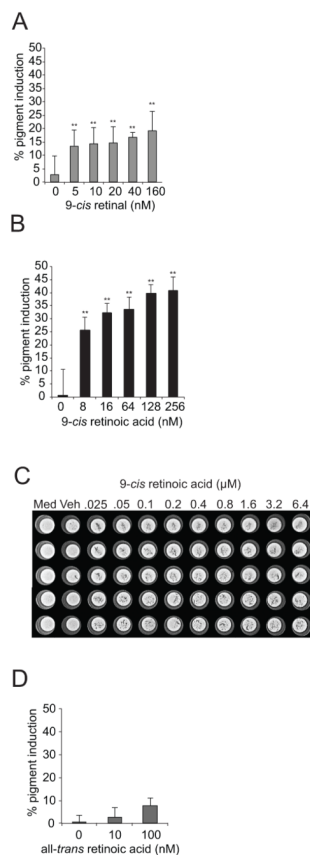t-test with a two-tailed normal distribution *versus* vehicle-treated control.

Figure 2. The metabolic product of 9-*cis* retinal oxidation, 9-*cis* retinoic acid, acts to stimulate melanin accumulation

(A) 9-*cis* retinal alone is sufficient to induce melanin accumulation. MNT-1 melanoma cells were briefly treated with the indicated doses of 9-*cis* retinal and then allowed to reach confluency. Melanin accumulation was measured as described in the methods section. **(B) 9-*cis* retinoic acid potently stimulates melanin accumulation.** MNT-1 melanoma cells were briefly treated with the indicated doses of 9-*cis* retinoic acid and allowed to reach confluency. Melanin accumulation was measured as described in the methods section **(C)** A light micrograph of a representative opaque-walled, clear-bottomed 96-well microtiter plate containing MNT-1 cells treated with 9-*cis* retinoic acid is shown. Med, wells containing only media; Veh, cells treated with vehicle. **(D) All-*trans* retinoic acid does not appreciably induce melanin accumulation.** MNT-1 melanoma cells were briefly treated with the indicated doses of all-*trans* retinoic acid and allowed to reach confluency. Melanin accumulation was measured as described in the methods section. *Data* shown are mean ± S.D. (*n*=6 as indicated by the *error bars*). *, $p < 0.05$ or **, $p < 0.01$ using a Student's paired t-test with a two-tailed normal distribution *versus* vehicle-treated control.
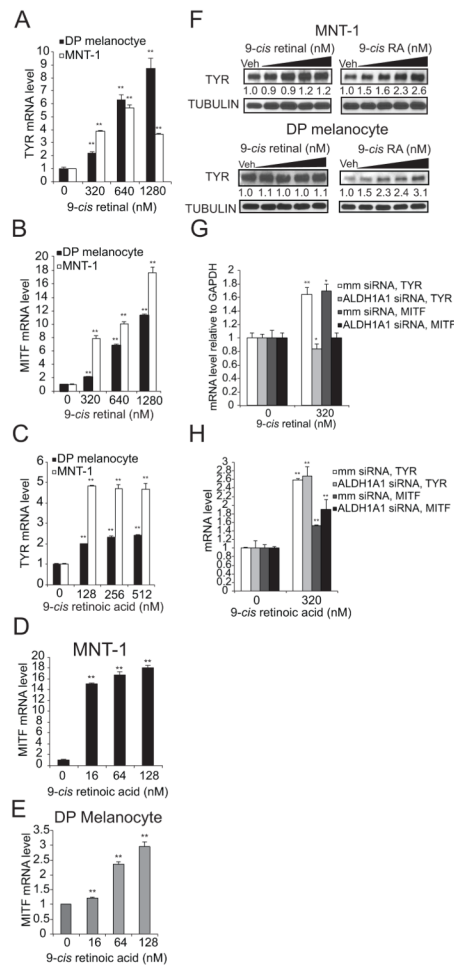
Figure 3. ALDH1A Regulates Melanogenesis by Converting 9-*cis* retinal to 9-*cis* retinoic acid (A–B) 9-*cis* retinal induces the accumulation of *TYR* and *MITF* mRNA independently of UVA. Primary melanocytes and MNT-1 cells were pre-incubated with 9-*cis* retinal at the indicated doses for 45 minutes. Relative accumulation of *TYR* (A) and *MITF* (B) mRNA was measured as described in the methods section. **(C–E) 9-*cis* retinoic acid induces the accumulation of *TYR* and *MITF* mRNA.** Primary melanocytes and MNT-1 cells were pre-incubated with 9-*cis* retinoic acid at the indicated concentrations for 45 minutes. Cells were harvested 20 hours post drug treatment, and the relative accumulation of *TYR* (C) and *MITF* (D, E) mRNA was quantified as described in methods. (**F) 9-*cis* retinoic acid but not 9-*cis* retinal induces the accumulation of TYR protein in both primary melanocytes and MNT-1 melanoma cells.** MNT-1 melanoma cells (upper panel) and primary melanocytes (lower panel) were pre-incubated with the indicated doses of 9-*cis* retinal or 9-*cis* retinoic acid for 45 minutes. 28 hours later, the relative accumulation of TYR protein was quantified by immunoblotting. The numerical values represent the relative intensity of the TYR band normalized to the Tubulin band (loading control) for each lane divided by the relative expression of the TYR band in the vehicle control. **(G–H) 9-*cis* retinal induces the accumulation of *TYR* and *MITF* mRNAs in an ALDH1A1 dependent manner.** MNT-1 melanoma cells transiently transfected with either mismatch control or ALDH1A1 siRNAs for 72 hours were briefly treated with the indicated doses of 9-*cis* retinal (G) or 9-*cis* retinoic acid (H). Cells were then harvested 20 hours post drug treatment and the relative accumulation of *TYR* and *MITF* mRNA was measured. All data are mean ± S.D. (n=3 as

indicated by the *error bars*). *, $p < 0.05$ or **, $p < 0.01$ using a Student's paired t-test with a two-tailed normal distribution *versus* vehicle-treated control.
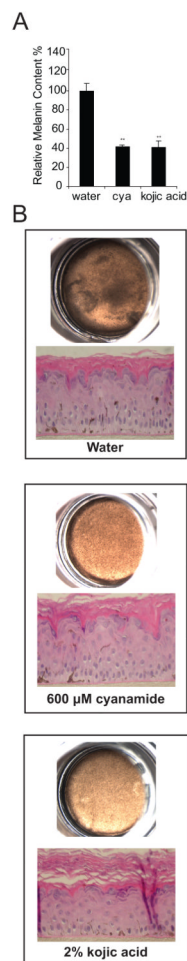
Figure 4. ALDH1A Controls Melanogenesis in Skin Equivalents
(A) Cyanamide inhibits melanin accumulation in skin equivalents as measured by a solvable melanin assay. Mattek darkly pigmented skin equivalents were incubated with 600 µM cyanamide, 2% kojic acid or water for 21 days. Melanin accumulation was quantified using a solvable melanin assay according to the manufacturer's protocol. **(B)** Equivalents were photographed using a dissecting microscope and then fixed and stained with a Fontana Masson silver stain to quantify the relative accumulation of melanin.