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DGAT1 Modulates Hair Cycling and Is Essential for Retinoid Homeostasis in the Skin

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DGAT1 Modulates Hair Cycling and Is Essential for Retinoid Homeostasis in the Skin

by

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DISSERTATION

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DEDICATIONS

To my parents, Jennifer and Hubert, for their unconditional love and support and for instilling in me the ambition to work hard. To my husband, Jethro, for having faith in me before I learned to have faith in myself.

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ABSTRACT OF THE DISSERTATION

***Dgat1* Modulates Hair Cycling and Is Essential for Retinoid Homeostasis in the Skin**

by

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Doctor of Philosophy in Biomedical Sciences

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Professor Robert V. Farese Jr., Thesis Advisor

Retinoids (retinol, or vitamin A, and its metabolic derivatives) play key roles in skin and hair biology. Both deficiency of retinoids and excess retinoids severely impair skin and hair function. The highly specific requirements for retinoids in the skin underscore the importance of maintaining retinoid homeostasis for normal skin biology. Nevertheless, the factors that regulate retinoid homeostasis in the skin are incompletely understood. Our laboratory recently showed that in *in vitro* assays, the microsomal enzyme encoded by *Dgat1*, an enzyme previously established to be important for triglyceride and energy metabolism, also possesses acyl CoA:retinol acyltransferase (ARAT) activity, an enzymatic activity that covalently joins retinol with a fatty acyl-CoA moiety to form retinyl esters. The goal of this dissertation was to determine whether the enzyme encoded by *Dgat1* functions as an ARAT enzyme in the skin and plays a role in maintaining retinoid homeostasis in the skin.

Because mice lacking *Dgat1* (*Dgat1*$^{-/-}$ mice) exhibit prominent alopecia, a well-established toxic consequence of excess retinoic acid (RA) signaling in the skin, I hypothesized that *Dgat1* functions to protect against retinoid toxicity in the skin by preventing the accumulation of unesterified retinol, which can drive excess RA biosynthesis.

First, I characterized the alopecia phenotype of *Dgat1*$^{-/-}$ mice and revealed *Dgat1* as a novel regulator of hair cycling. *Dgat1*$^{-/-}$ mice exhibit altered hair cycling characterized by a prolonged first anagen phase and the precocious onset of the second anagen phase at ~8 weeks of age. Consistent with the lack of evidence for hair cycle arrest, the alopecia of *Dgat1*$^{-/-}$ mice does not result from an arrest in hair growth but from excessive hair shedding. Because the onset of excessive shedding at 9 weeks of age coincides with the precocious onset of the second anagen phase, the alopecia manifests as a cyclical pattern of hair loss followed by immediate hair re-growth, a pattern that persists in older animals. Analysis of *Dgat1* expression in the skin during the depilation-induced hair cycle showed highest levels of *Dgat1* mRNA detected during telogen. This data, together with the increased propensity of *Dgat1*$^{-/-}$ follicles to be in anagen, suggest that *Dgat1* likely plays a role in maintaining hair follicles in the quiescent telogen phase.

Next, I examined retinol metabolism in skin of *Dgat1*$^{-/-}$ mice. I found that *Dgat1* is the principal ARAT in the skin responsible for 90% of the total ARAT activity. In the absence of *Dgat1*, elevated levels of retinol and all-*trans*-RA, as well as increased RA signaling were detected in skin. The excess RA in the skin is the cause of alopecia in *Dgat1*$^{-/-}$ mice because dietary retinol deprivation reduced skin RA levels and prevented the alopecia. Selective deletion of *Dgat1* in the epidermis was sufficient to cause alopecia, indicating the retinol acyltransferase function of DGAT1 is epidermis-autonomous. *Dgat1*$^{-/-}$ skin was also

more susceptible to topical retinol-induced toxicity suggesting *Dgat1* protects against the accumulation of unesterified retinol in the skin. From these findings, I concluded that the enzyme encoded by *Dgat1* functions as a retinol acyltransferase in the epidermis where it plays a pivotal role in maintaining retinoid homeostasis, likely by restricting RA biosynthesis through controlling retinol levels. The work presented in this dissertation highlights previously unrecognized roles for DGAT1 as an ARAT enzyme in the skin and as a novel regulator of hair cycling.

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CHAPTER I.

Introduction

Overview

Retinoids (retinol, or vitamin A, and its derivatives) play key roles in skin and hair biology.[1,2] Deficiency of retinoids results in thinning of the epidermis and atrophy of hair follicles and sebaceous glands.[1] On the other hand, excess retinoids induce epidermal hyperplasia, suppress keratinization, inhibit sebocyte proliferation and differentiation, impair barrier function, and alter hair growth.[1,3,4] The pleiotropic effects of retinoids make them efficacious pharmaceuticals for the treatment of dermatological diseases, including cystic acne, psoriasis, and other keratinization disorders.[4-6] However, the complex effects of retinoids also underscore the importance of retinoid homeostasis in normal skin biology. Despite the importance of retinoids in skin, the regulatory mechanisms that maintain retinoid homeostasis are incompletely understood. In this thesis, I conduct studies that further our understanding of the mechanisms that regulate retinoid metabolism and maintain retinoid homeostasis in the skin.

Retinoid Action in the Skin

Mechanism of action. Retinol (vitamin A), an essential micronutrient that must be acquired from the diet, exerts most of its biological effects after two consecutive oxidation reactions to form retinoic acids (RA).[7] With the exception of the visual and immune functions of retinol, the RA isomers, all-*trans*- and 9-*cis*-RA, are the major effectors. [8] Furthermore, all-*trans*-RA can fulfill almost all the biological functions of retinol.[8] RA exerts its pleiotropic effects by acting as ligands for nuclear receptor transcription factors, retinoic acid receptor (RAR) and retinoid x receptor (RXR), that belong to the nuclear receptor superfamily.[9] Several

isoforms of RAR and RXR exist including RARα, β, and γ, which bind both all-*trans*-RA and 9-*cis*-RA, and RXRα, β, and γ, which bind 9-*cis*-RA only.[9]

Liganded RAR and RXR heterodimerize and bind DNA at specific sequences known as RA response elements (RARE).[10] RARs preferentially bind RAREs with direct repeats of conserved nucleotide hexamers separated by two, four, or five nucleotides (DR2, DR4, or DR5).[10] Whereas RXRs bind DR1 motifs.[10-12] Currently over 500 genes are postulated to be regulated by RAR/RXR receptors.[13] Unliganded RAR/RXR heterodimers also bind RAREs but are associated with co-repressors and suppress gene expression.[14]

RXRs also homodimerize and are obligate heterodimeric partners for other nuclear receptors including thyroid hormone (TR), vitamin D3 (VDR), liver X (LXR), peroxisome proliferators (PPARs) and several orphan receptors.[10] PPAR/RXR heterodimers bind to directs repeats separated by one nucleotide (DR1), while LXR/RXR heterodimers activate transcription at direct repeats separated by four nucleotides (DR4). Whereas, ligand activation of TR and RAR are required to induce heterodimerization with RXR[15], heterodimerization of PPAR/RXR and LXR/RXR can be induced by activation of either receptor unit.[11,16] Therefore 9-*cis*-RA is capable to activating a number of signaling pathways besides RAR signaling but the endogenous presence of 9-*cis*-RA is still controversial.[17]

Retinoid receptors in skin. The predominant retinoid receptors detected in the epidermis are RXRα and RARγ, but RXRβ and RARα expression have also been detected.[2] The precise functions of these retinoid receptors in the epidermis are incompletely understood. Germ-line disruptions of retinoid receptors have revealed little of their function in the epidermis due to functional redundancy. RARα, RARγ, and RXRβ were found to be dispensible for

epidermal homeostasis, whereas RXRα deficiency and compound germ-line disruption of RARα and RARγ lead to lethality before embryonic day (E) 14.5 and E11.5, respectively.[2]

Epidermis-specific ablation of retinoid receptors revealed that both RARα and RARγ are dispensable for epidermal development and homeostasis.[18,19] RXRα deficiency in the epidermis however leads to a delay in postnatal hair growth and epidermal maturation. [3,20,21] Progressive alopecia begins to develop in *Rxrα*-deficient ($Rxr\alpha^{-/-}$) mice at 4-5 weeks and by 12-16 weeks of age, mice are 80% and 30% bald on the ventral and dorsal skin, respectively. The progressive alopecia is likely caused by a defect in anagen initation as the majority of mutant hair follicles failed to initiate anagen after depilation at 20 days postpartum (dpp). Hair follicles eventually degenerate into utriculi by 12 weeks of age, and dermal cysts are also prominent by 12-16 weeks of age and become widespread all over the body with increasing age. Melanosome defects were also observed. In alopecia areas, hyperplastic interfollicular epidermis and increased dermal cellularity were evident. This was accompanied by the infiltration of inflammatory cells including increased numbers of Langerhan cells in the suprabasal and basal layers of the epidermis, CD3 positive T-cells in the epidermis and dermis, CD4 positive T-cells in the dermis, utriculi, and dermal cysts, and macrophages and mast cells in the epidermis and dermis, without any signs of bacterial or fungal infection. Increased expression of the pro-inflammatory stimuli, ICAM1, was detected in the mutant epidermis and dermis. [20,21] The inflammation in the mutant skin is unlikely to be a secondary effect of hair follicle defects because $Vdr^{-/-}$ mice[21,22] which exhibit a very similar alopecia as $Rxr\alpha^{-/-}$ mice but without the epidermal alterations, exhibit very little skin inflammation. Because RXRα heterodimerizes with a number of other nuclear receptors (VDR and PPARs) that are expressed in the epidermis and hair follicles,

the effects of RXRα deficiency may result from the disruption of signaling pathways mediated by a number of other nuclear receptors. The alopecia of *Rxrα*$^{-/-}$ mice is likely the result of the loss of VDR/RXRα function in the skin.

To overcome the functional redundancy between RARα and RARγ, transgenic expression of dominant-negative (dn) receptors were targeted to the basal and suprabasal keratinocytes of the epidermis. Expression of dn RARα, which inhibited the endogenous activity of RARα, β, and γ, in suprabasal keratinocytes resulted in defects in lipid deposition by differentiated keratinocytes and loss of a functional lipid barrier.[23-25] The only epidermal phenotype resulting from the expression of dn RARγ in suprabasal keratinocytes was a "shiny" appearance of neonatal skin which normalized by adulthood. [23-25] However, because dn receptors can interfere with, through sequestration, signaling pathways mediated by other nuclear receptors that heterodimerize with RXR and could repress the expression of genes that are not "normal" targets of co-repressor associated unliganded RXR/RAR heterodimers, the abnormalities exhibited by these transgenic models may not reflect physiological roles of RA in the epidermis.

Cutaneous effects of pharmacologic retinoids. Albert Kligman, in a number of controversial studies conducted in the 1960s, first discovered the profound effects of retinoids on sebaceous gland function.[26] Despite the questionable methods employed, this discovery revolutionized the treatment of acne and retinoids are currently still the most efficacious acne therapy available. 13-*cis*-RA, also known as isotretinoin, is the most effective retinoid for reducing sebaceous gland size and secretion. It decreases the proliferation of basal sebocytes and suppresses sebum production up to 90% by inhibiting lipid synthesis and late-stage

differentiation of human sebocytes in culture.[27] The anti-proliferative and anti-lipogenic effects of isotretinoin are thought to be mediated by intracellular isomerization to all-*trans*-RA and activation of RAR because a significant elevation in all-*trans*-RA was seen specifically in sebocytes and not keratinocytes treated with 13-*cis*-RA and antagonism of RAR by a pan-RAR antagonist blocked these effects.[27] Furthermore, all-*trans*-RA has been directly shown to inhibit proliferation and decrease lipid synthesis in human sebocytes in culture. [27] It was recently reported that 13-*cis*-RA acid also induces cell cycle arrest and apoptosis of human sebocytes in culture.[28] The effects of isotretinoin on cell cycle arrest and apoptosis, however, were not blocked by the pan-RAR antagonist, suggesting these effects are mediated by a RAR-independent mechanism.[28]

Unfortunately, the use of systemic or topical retinoids for the treatment of acne produces a number of undesired effects on the skin. [29] Retinoids stimulate proliferation of keratinocytes in the basal layer of the epidermis. Increased proliferation of basal keratinocytes accompanied by vectoral migration toward the skin surface lead to acanthosis (benign thickening of the spinous layer of the skin) and hypergranulosis.[29] Retinoids also induce shedding of desmosomes by the spinous layer, decrease tonofilaments, enhance keratinocytes autolysis, and intercellular deposition of glycoconjugates leading to poor cohesion of the stratum corneum.[29] These effects of retinoids manifest in severe skin scaling and peeling. These undesired effects of retinoids, also referred to as epidermal hyperplasia and stratum corneum fragility, have fortunately been harnessed for the successful treatment of a number of keratinization disorders such as psoriasis, ichthyosis, and Darier's disease.[30,31] Unfortunately, however, these effects of retinoids are transient during long-term retinoid therapy as retinoid resistance eventually develops. Two postulated mechanisms of retinoid

resistance include the RA-mediated induction of cellular-retinoic acid binding proteins which lower the plasma and intracellular levels of active RA by binding and directing RA for catabolism[32], and the RA-mediated induction and/or constitutive overexpression of a P-glycoprotein encoded by the multidrug resistance gene (MDR1), which reduces intracellular RA levels by active transport of intracellular RA out of target cells.[33-35]

Retinoids also exert pleiotropic and paradoxical effects on hair growth. On one hand, a major effect of systemic retinoids, or hypervitaminosis A (vitamin A toxicity), is substantial diffuse hair loss.[36,37] In fact, retinoid-induced hair loss is the most common cause of the cessation of retinoid therapy, by both men and women, despite positive therapeutic responses.

Hair is produced by the hair follicle, a complex mini-organ that undergoes periodic cycles of growth and involution. During hair follicle cycling, dramatic morphological transformations occur in the hair follicle as it transitions from a resting (telogen) phase to a growth (anagen) phase, with rapid proliferation of follicular keratinocytes and elongation and thickening of the hair shaft, to an apoptosis driven regression (catagen) phase.[38] Studies of scalp hair from patients on systemic eretinate therapy suggest that hair loss is provoked by the shortening of the anagen phase/premature entry into telogen (telogen effluvium), an arrest at the onset of anagen, and defective anchoring of the hair shaft during telogen leading to premature hair shedding.[37] A study using cultured human anagen VI hair follicles showed that all-*trans*-RA inhibited hair shaft elongation and induced premature catagen entry by inhibiting proliferation and stimulating apoptosis of hair follicle matrix keratinocytes.[39] These effects of all-*trans*-RA resembled the effects of transforming growth factor- β2 (TGF-β2), a major factor implicated in the initiation of catagen[40], and was partially reversed with

the addition of anti-TGF-β antibody.[39] In accordance, *all-trans*-RA treatment of cultured follicles upregulated intrafollicular TGF-β2 expression.[39] Hair loss is also a major toxic consequence of hypervitaminosis A in mice[41], however, the mechanism of retinoid-induced hair loss in mice has not been studied extensively. Whether the characteristics of retinoid-induced hair loss in mice resemble those in humans is unknown.

Paradoxically, retinoids also exert growth promoting effects on human and murine hair follicles.[42,43] Dermatologists observed increased vellus facial hair in patients with cystic acne treated with oral isotretinoin therapy. This observation led to several studies on the effects of topical tretinoin on hair growth and re-growth in patients diagnosed with androgenetic alopecia.[43] They found that topical application of tretinoin on human scalp exerted anagen promoting and prolonging effects which were further enhanced by the addition of minoxidil.[42-45]

The anagen promoting and prolonging effects of tretinoin were further supported by findings in mice. Topical retinoids administered to telogen hair follicles of the dorsal skin of mice rapidly stimulated anagen initiation and prolonged the length of the resulting anagen phase compared with the length of spontaneous- or mechanically (depilation)-induced anagen.[42] The precise physiological function of retinoids in the regulation of hair cycling is currently unclear. However, several important molecules of retinoid metabolism [*e.g.*, CRABPII, retinol dehydrogenase (Dhrs9), and aldehyde dehydrogenases (Aldh1a1–3)] and retinoid signaling (RARβ and RARα) exhibit cycle-dependent spatiotemporal expression patterns in the hair follicle, suggesting that local retinoid production and signaling is required in different structures of the follicle at each stage of the cycle.[46,47]

Although genetic mutation studies have shown that RARγ is dispensible for the physiologic maintenance of epidermal homeostasis, studies show that RARγ and RXRα are required for the pharmacologic effects of retinoids in the skin.[3,41] Mice with a germ-line disruption of RARγ exhibited marked resistance to the gross toxic effects (skin scaling and hair loss) of fourfold higher doses of systemic RA than wild-type mice.[41] Epidermis-specific ablation of RARγ or RXRα also conferred marked resistance of the epidermis to topical retinoid-induced hyperproliferation, and compound ablation of RARγ and RXRα in the epidermis conferred complete resistance to the effects of topical retinoids.[3] Despite significant progress in dissecting the role of retinoid receptors in retinoid-induced hair loss and epidermal toxicity, the role of retinoid receptors in the mechanism of retinoid-induced hair growth is largely unknown.

Retinol Metabolism in the Skin

Dietary retinol absorption, transport, and uptake by skin. Retinol can be acquired from the diet as provitamin A carotenoids, such as β-carotene, from plant sources or as retinyl esters – retinol covalently joined to a fatty acyl-CoA moiety – from animal organs and fish liver.[48] Within enterocytes, some β-carotene passes though unchanged into the lymph, then circulation, but a proportion is cleaved symmetrically by a specific enzyme 15,15'-dioxygenase to yield two retinal molecules, which are then reduced to retinol by an intestinal microsomal retinal reductase.[49] Some β-carotene is cleaved asymmetrically but this process yields less retinol.[49] The retinyl esters derived from the consumption of animal organs are first hydrolyzed by pancreatic lipase in the intestinal lumen and taken up by enterocytes as unesterified retinol. Once absorbed by enterocytes, both pools of retinol are bound by

cellular-retinol binding protein II (CRBPII, the predominant CRBP expressed by enterocytes) which facilitates the esterification of retinol by directing retinol to the enzyme lecithin:retinol acyltransferase (LRAT). LRAT esterifies CRBP-bound retinol with a fatty acyl moiety donated from the phospholid, lecithin. Another retinol acyltransferase activity, the acyl-CoA:retinol acyltransferase (ARAT) activity, which recognizes "free" retinol, *i.e.* retinol not bound by CRBP, and utilizes fatty acyl-CoA as co-substrate, is also detected in the intestine. However, studies show that the genetic ablation of CRBPII reduced hepatic retinol stores by ~40% [50] and that intestinal retinol absorption is impaired in mice lacking LRAT[51], suggesting LRAT mediated retinol esterification is the predominant mechanism for retinyl ester formation in the intestine. The retinyl esters are then packaged into chylomicron particles and secreted into the lymphatics, and eventually blood circulation, for delivery to major tissues of retinol storage and retinoid action. Some retinol can bypass esterification and enter circulation bound to albumin.[51]

Approximately 25-30% of the dietary retinol in chylomicrons is removed by extrahepatic tissues in a single pass.[51-53] Prior to uptake by extrahepatic tissues, chylomicron derived retinyl esters are first hydrolyzed to retinol by lipoprotein lipase. [52,53] Whether retinol enters cells via passive diffusion or receptor-mediated internalization is currently unclear. The remaining 70-75% of excess retinyl esters in remnant particles are taken up by the liver, the major storage site for retinol.[52,53] In the liver, retinyl esters are first hydrolyzed by retinyl ester hydrolase into retinol. Retinol is then taken up by perisinusoidal stellate cells, which express both LRAT and CRBP, and is re-esterified and stored as retinyl esters. [52,53] These retinyl esters can be mobilized during fasting and secreted back into circulation in a complex with retinol-binding protein (RBP) and its cofactor, transthyretin, a serum protein

named for its ability to bind and transport - simultaneously but independently - thyroid hormone and the retinol-RBP complex, to meet the needs of extrahepatic tissues.[52,53] Recent studies revealed that a significant amount of dietary retinol can also be stored within the white adipose tissue (WAT) and mobilized during dietary retinoid insufficiency.[51] The ability to store excess retinol as retinyl esters in the liver, as well as in other tissues such as the WAT and lung, prevent retinol from non-specific metabolism and excretion and protects against the development of retinoid deficiency. Mice lacking CRBPI exhibited a 50% reduction in retinol stores in hepatic stellate cells due to a 6-fold faster turnover of retinol and decreased retinyl ester synthesis.[54] Furthermore, mice lacking CRBPI or LRAT exhibit increased susceptibility to retinoid deficiency when retinoids are removed from the diet.[54,55] Without the ability to store and protect unused retinol as retinyl esters, the retinoid status of the animal would be extremely tenuous and highly dependent upon dietary retinoid intake.

The mechanism of RBP-retinol uptake by extrahepatic tissues is currently under debate. Evidence for both mechanisms of passive diffusion and receptor-mediated internalization exist.[5] Genetic disruption of RBP4, the sole retinol specific carrier protein in plasma, reduced plasma retinol levels in mice to one-tenth that of wild-type mice, reduced retinol and retinyl ester levels in the retina, and caused retinol accumulation in the liver.[56] However, these mice are phenotypically normal when a fed retinoid-sufficient diet suggesting that although RBP4 is necessary for mobilizing retinol from the liver and for retinol uptake by the retina, RBP4 is not essential for retinol uptake in most tissues including skin.[56]

There is also abundant evidence supporting the existence of a specific cell surface receptor for RBP. Evidence of a receptor in the retinal pigment epithelium (RPE) was first

reported in the 1970s. Currently, there is evidence for the existence of a RBP receptor on other tissues or cells types, including the placenta[57-61],choroid plexus[60,62], testis[60,63], and macrophages.[64] STRA6, a multitransmembrane domain protein, was recently identified as a specific membrane receptor for RBP4.[65] This protein is widely expressed during embryonic development and in adult mice.[65] Germ-line and tissue-specific disruption of STRA6 in mice will be necessary to access the contribution of this receptor to retinol uptake by cells and tissue and to assess whether this receptor has other functions besides mediating retinol uptake.

Overview of intracellular retinol metabolism. It is postulated that once taken up by different cells in the skin, the majority of retinol (from RBP4 or chylomicrons) in the cytoplasm is bound by CRBP and directed to different enzymes necessary for its metabolism.[66] CRBP-retinol can undergo oxidation to form bioactive retinoids, such as all-*trans*-RA or 9-*cis*-RA, which can serve as ligands for retinoid receptors or be further hydroxylated to form polar metabolites for excretion. CRBP-retinol in excess of cellular need can also be esterified to form retinyl esters and stored within intracellular lipid droplets. Whether intracellular "free" retinol exists and serves as a physiological substrate for retinol metabolism is a controversial subject and is partly addressed by studies described in this thesis.

Cellular retinol binding proteins. CRBPs are members of the fatty acid-binding protein superfamily and are highly expressed in most tissues of animals. There are currently three known isoforms of CRBP, type I, II, and III. Whereas CRBPI and II bind both retinol and retinal, CRBPIII only binds retinol. CRBPI is the most widely expressed of the three

isoforms and is found in almost all tissues of the developing and adult mice, including the epidermis.[67-72] During development, CRBPI is specifically expressed in several tissues including motor neurons, spinal cord, liver, lung[73-76], and the placenta.[77,78] CRBPII expression is restricted to the yolk sac during gestation and the liver during late gestation and right after birth. CRBPII is, however, steadily expressed throughout life, in the villus-associated enterocytes involved in retinol uptake in the intestine.[79] Although CRBPI and CRBPII are both detected in the fetal liver, they are not co-expressed.[80] CRBPI is expressed in the sinusoids and blood vessels while CRBPII is expressed solely in hepatocytes suggesting unique functions for CRBPI and II. [80] CRBPIII expression is restricted to the heart, adipose tissue, muscle, and mammary tissue of postnatal mice.[81-83]

Because copious amounts of "free" retinol can disrupt normal cellular activities by non-specifically incorporating into cellular membranes and by driving excess RA biosynthesis, it has been proposed that a physiologic role of CRBP is to protect cells against these potentially detrimental effects.[84] Many enzymatic activities of retinol metabolism, such as the LRAT activity[80,85] and the retinol and retinal dehydrogenase activities, only recognize CRBP-bound retinol as substrate in *in vitro* assays suggesting CRBP may be essential for directing intracellular retinol to enzymes necessary for its metabolism. Overexpression of CRBPI in HL60 cells and hepatic stellate cells enhance retinol uptake.[86] Studies have also shown that holo-CRBP stimulates retinol esterification by LRAT while apo-CRBP stimulates retinyl ester hydrolysis. Apo-CRBPI also inhibits RA biosynthesis from CRBPI-bound retinol suggesting that relative levels of apo- and holo-CRBPI control the oxidation or conservation of retinol according to retinol availability.[87]

The major CRBP in the skin is CRBPI and expression is detected in the keratinocytes of the epidermis and hair follicles, dermal fibroblasts, and the intradermal adipose tissue layer of the skin. Topical application of RA stimulates CRBPI expression.[88] A RARE characterized by direct repeats of motif 5'-GGTCA-3' separated by 2 nucleotides (DR2) was found in the CRBPI promoter and shown to be necessary for RA induction of CRBPI expression.[89]

Surprisingly, *CrbpI*$^{-/-}$ mice fed a retinoid-enriched diet are healthy and fertile. They do not exhibit any congenital defects associated with RA deficiency, suggesting CRBPI is dispensible for retinoic acid biosynthesis.[54] However, retinyl ester levels in hepatic stellate cells were reduced by ~50% due to a decrease in retinyl ester synthesis and a 6 fold increase in retinol turnover, thus making these mice vulnerable to developing retinoid-deficiency on a retinol-deficient diet.[54] Therefore, CRBP appears necessary for directing retinol to the LRAT enzyme for retinyl ester synthesis and storage in the liver. Despite the defect in hepatic retinyl ester storage, serum retinol levels were maintained and no retinoid-deficiency or toxicity associated defects were reported in the epidermis of *CrbpI*$^{-/-}$ mice on the retinoid-enriched diets. [54] Overexpression of a human CRBPI in mice also did not alter retinoid homeostasis and did not result in any phenotypic defects associated with retinoid-toxicity (from increased targeting of retinol for RA biosynthesis) or retinoid-deficiency (from sequestration of retinol in the form of retinyl esters in storage tissues).[90]

No epidermal defects were reported for CRBPII and CRBPIII null mice as expected because expression of these two isoforms of CRBP is not detected in the epidermis. *CrbpII*$^{-/-}$ mice grow normally and are fertile on a retinoid-enriched diet.[50,91] However, CRBPII deficiency in mice results in increased neonatal mortality on a retinol-marginal diet because

retinol uptake by the intestine of *CrbpII*$^{-/-}$ mice is greatly impaired leading to a ~40% decrease in retinyl ester stores in the liver.[50] Thus CRBPII expression in the intestine is required for optimal retinol uptake when dietary retinol is limiting.

CrbpIII$^{-/-}$ mice are healthy, viable, and fertile but exhibit impaired retinoid incorporation into milk due to impaired retinol utilization by LRAT.[91] Interestingly, CRBPI was elevated in the adipose tissue, muscle, heart, and mammary tissue of *CrbpIII*$^{-/-}$ mice and CRBPIII was elevated in the tissues that normally express CRBPIII in *CrbpI*$^{-/-}$ mice suggesting CRBPI and CRBPIII share similar physiologic actions and can compensate for each other to maintain retinoid homeostasis.[91] However, during times of increased retinoid demand (as in lactation), the compensation is incomplete. [91]

Retinoic acid biosynthesis. The oxidation of retinol to retinoic acids fulfills the signaling needs of most tissue, however, the generation of retinoic acids is also an intermediate step in the mechanism of retinol excretion from cells. Retinol is endogenously converted to retinoic acids via two consecutive enzymatic oxidation reactions, with retinal (retinaldehyde) as an intermediate. *In vitro* studies show that cytosolic medium-chain alcohol dehydrogenases (ADH) as well as membrane-bound short-chain dehydrogenase/reductase (SDR) can oxidize all-*trans*-retinol to all-*trans*-retinal (retinaldehyde), the first oxidation step in RA biosynthesis, in a reversible reaction.

Currently four major classes of ADH enzymes have been identified in mice, class I (ADH1), class II (ADH2), class III (ADH3), and class IV (ADH4), all of which recognize "free" retinol. Most studies have focused on ADH1, ADH3, and ADH4 because these are the most highly conserved ADHs in all mammalian species examined.[92,93] The ADH3 enzyme is

ubiquitously expressed and both ADH1 and ADH4 are expressed in the epidermis as well as other retinoid–responsive epithelia including the male reproductive tract, stomach/esophagus, and the gastrointestinal tract.[94-97] Mice lacking ADH1, ADH3, or ADH 4 develop normally and do not exhibit any postnatal defects.[98] Mice lacking both ADH1 and ADH4 were also viable and did not exhibit any developmental or postnatal defects.[99] Overlapping roles and sites of expression of ADH1 and ADH4, as well as the presence of short-chain dehydrogenase/reductase enzymes, may explain the lack of any physiological effects in the double knockout mice. Therefore, it is currently unclear which and if the ADH1 and ADH4 enzymes oxidize retinol in the epidermis for the biosynthesis of RA for signaling or excretion. The expression patterns of ADH1 and ADH4 in the epidermis and epidermal appendages are also unknown.

So far at least ten cDNA clones encoding microsomal members of the SDR family have been isolated. Members of the SDR family can catalyze the conversion of all-*trans*-retinol and various *cis*-retinol isomers bound to CRBP. Each member shows a unique pattern of catalysis with various retinoids. Two human epidermal SDR family members, hRDH-E and hRDH-E2, have been identified[100,101]. Recently, hRDH-E2 transcripts have been detected in the sebaceous and sweat glands, and in the matrix cells and the Huxley cells of the inner root sheath of the infundibulum of the hair follicle.[101]

The second oxidation step in RA biosynthesis, conversion of retinal into bioactive retinoic acids, is irreversible and carried out by aldehyde dehydrogenases. Currently there are three known aldehyde dehydrogenase enzymes Aldh1a (1-3). Immunolocalization studies in mouse skin show that Aldh1a2 and Aldh1a3 are expressed in the epidermis and also exhibit hair cycle-dependent spatiotemporal expression patterns in the hair follicle which

suggest that local retinoid production and signaling is required in different structures of the hair follicle at all stages of the hair cycle.[46]

Retinoic acid metabolism. The enzymatic activity mainly responsible for the metabolism of RA to inactive metabolites is the cytochrome P450 (CYP)-dependent 4-hydroxylase that converts RA to 4-*hydroxy*-RA (4-OH-RA) metabolites.[5] 4-OH-RA exhibits significantly less biological activity than RA and is excreted much faster from cells than RA.[102-104] Oral administration of R115866, an inhibitor of CYP-mediated RA metabolism, markedly elevated endogenous RA levels in plasma, skin, WAT, kidney, and testis and induced similar toxic effects as RA[105], underscoring the importance of RA metabolism for the regulation of retinoid activity.

Interestingly, the 4-hydroxylase activity that is induced in the epidermis by the topical application of different isomers of RA (all-*trans*-, 9-*cis*-, and 13-*cis*-) only metabolizes all-*trans*-RA. However, this activity still generates an increase in 4-OH-metabolites in the epidermis regardless of the isomer applied.[106,107] It is likely that 9-*cis*- and 13-*cis*-RA isomerize rapidly to all-*trans*-RA in the epidermis and are metabolized by the all-*trans*-RA 4-hydroxylase.

4-OH-RA can also be further metabolized to 4-oxo-RA in keratinocytes in a reaction that requires NAD. Studies suggest that 4-OH-RA oxidizes to 4-oxo-RA spontaneously depending on the level of RA and induction of the RA 4-hydroxylase activity. 4-oxo-RA is the most characterized metabolite of RA and has been found to exhibit retinoid receptor binding activity but is half as biologically active as RA.[108,109]

An all-*trans*-RA 4-hydroxylase in epidermal keratinocytes has been cloned and designated CYP26.[110,111] However, the contribution of CYP26 to RA metabolism in the skin has not been established.

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Cellular retinoic acid binding proteins. Besides RAR and RXR ligand-inducible nuclear receptor transcription factors, another class of intracellular proteins binds RA with high affinity, the cellular retinoic acid binding proteins. There are two homologous proteins, CRABPI and CRABPII that display different expression patterns suggesting that they serve different functions in the biology of RA or that they allow for accommodating different requirements for RA in different tissues. In the adult, CRABPI is expressed almost ubiquitously, whereas CRABPII expression is limited. CRABPII expression has been detected in the liver[112], the skin[113], uterus, ovary[72,114], and in the choroid plexus[115]. Both CRABPs are found in the cytoplasm as well as the nucleus.[116]

Interestingly, despite the high conservation of CRABPs, mice in which both of the genes have been disrupted appear essentially normal.[117] However, many *in vitro* studies suggest that CRABPI and CRABPII perform unique functions *in vivo.* The rate of RA degradation in F9 teratocarcinoma cells is reported to be enhanced upon overexpression of CRABPI.[118,119] Furthermore, the sensitivity of F9 cells to RA induced differentiation is inversely correlated to the cellular level of CRABPI.[118,119] However, in COS-7 and CV-1 cells, CRABPI did not inhibit the transcriptional activity of RA.[120] The difference in the effect of CRABPI on the transcriptional activity of RA may be dependent on whether the cell type expresses RA-metabolizing enzymes. Such enzymes do exist in F9 cells[118,119] but their presence in COS-7 and CV-1 cells are unknown. Therefore, CRABPI appears to be

important for moderating RA action by facilitating its catabolism and/or by sequestering RA and preventing its interaction with retinoid receptors.

On the other hand, overexpression of CRABPII in COS-7 cells enhanced RA induced RAR-mediated transcriptional activity and overexpression of CRABPI had no effect.[120] Findings from several studies suggest that CRABPII is important for delivering RA to the RAR and facilitating RA signaling. CRABPII facilitates the transfer of RA to the RAR receptor by a mechanism that involves direct interaction between CRABPII and the RAR receptor, whereas transfer of RA from CRABPI to RAR involves dissociation of the RA from CRABPI.[120] Also, ectopic expression of CRABPII in mammary carcinoma cell lines enhanced RA transcriptional activity[121] and knock down of CRABPII expression by antisense construct in SCC25 cells reduced their sensitivity to RA-mediated inhibition of proliferation.[122]

Topical RA application induces CRABPII but not CRABPI mRNA expression in human and mouse skin.[123,124] RA-induced CRABPII mRNA expression involved two cooperating RAREs, RARE1 (DR1) and RARE2 (DR2), in the promoter of the CRABPII gene that mediate transcriptional transactivation by RAR/RXR heterodimers.[125]

Retinol esterification. Esterification of retinol with fatty acyl moieties to form retinyl esters also contributes to retinol metabolism in the skin.[126]. Retinyl esters are the most abundant form of retinol found in tissues. Retinol esterification provides a means to maintain intracellular stores of retinol and to prevent toxicity from excessive levels of unesterified retinol.

As mentioned previously, two major retinol esterification activities have been identified. One activity is mediated by LRAT which utilizes lecithin (phosphotidylcholine) as the fatty acyl donor to esterify retinol that is bound to CRBPs.[85] The other retinol esterification activity is the ARAT activity which recognizes "free" retinol, *i.e.* retinol not bound by CRBP, and utilizes fatty acyl-CoA as the co-substrate.

The enzyme responsible for the LRAT activity has been cloned[127]and *Lrat*$^{-/-}$ mice recently generated.[51,55,128] *Lrat*$^{-/-}$ mice develop normally but under closer examination exhibit reduced levels of retinyl ester stores in certain tissues and defects in visual and testicular function resulting from local retinoid deficiency. Histological and electron microscopy analysis of the eyes of *Lrat*$^{-/-}$ mice showed that the rod out segments were shortened by 30% but other neuronal layers appeared normal.[128] Scotopic and photopic electroretinograms and papillary constriction analysis revealed severely attenuated rod and cone function in young *Lrat*$^{-/-}$ mice.[128] Defects in testicular development and spermatogenesis characterized by testicular hypoplasia/atrophy and oligospermia with the absence of spermatogenic epithelium, dilated seminiferous tubular lumens, and occasional luminal giant cells were also detected, indicating LRAT function is necessary for normal testicular development and function.[55] Intestinal retinol absorption was greatly reduced in *Lrat*$^{-/-}$ mice and only trace retinyl ester levels were detected in a number of major retinol storage tissues including the liver and lungs which make them vulnerable to the development of retinoid-deficiency when fed a retinoid-deficient diet.[51,55] However, when fed a retinoid-sufficient diet, serum retinol levels are maintained in *Lrat*$^{-/-}$ mice.[51,55] These findings indicate that LRAT functions as the predominant retinol esterification enzyme for intestinal retinol absorption and maintaining retinol stores in tissues. Although retinoid levels were not

measured in *Lrat*$^{-/-}$ skin, gross defects were not reported. Interestingly, retinyl esters levels were normal in the WAT of *Lrat*$^{-/-}$ mice indicating the existence of alternative mechanisms for retinol esterification.[51,55] This esterification is likely mediated by ARAT.[66]

The ARAT activity was first discovered in the microsomal fraction of liver, intestine, and kidney homogenates in the 1950s[129]. This discovery led to the rapid identification of ARAT activity in many other tissues of mice, rats, and humans where retinyl esters were abundant. By the 1980s, ARAT activity in the RPE[130], liver[131], intestinal mucosa[129], lactating mammary gland[132], and epidermis[133] had been extensively studied. These studies revealed regulation of the ARAT activity in the liver and intestine by dietary retinol availability and feeding status. ARAT activity was shown to be induced in the intestine and liver of rats fed large doses of dietary retinol but not significantly affected by dietary retinoid-deficiency. [134] Also fasting was found to induce ARAT activity in the intestine, whereas high-fat feeding exerted the opposite effect.[135,136] Similar to the liver and intestine, epidermal ARAT activity was also found to be regulated by retinol availability. In mouse epidermis, ARAT activity was increased by treatments that elevated epidermal unesterified retinol levels including topical retinol, topical retinaldehyde, which is rapidly reduced to retinol in the epidermis, and UVB irradiation, which stimulates a large influx of retinol from the plasma into the epidermis.[137]

Up until the late 1980s, the ARAT activity was considered a major retinol esterification activity *in vivo*, however, after the identification of CRBPs and the discovery that CRBP-bound retinol participates in an acyl CoA-independent mechanism of retinol esterification in the rat small intestine, the role of the ARAT activity in retinol esterification was quickly reduced to a minor participant. The discovery that CRBPs were highly

expressed in most tissues and that their major function was to deliver retinol to retinol metabolizing enzymes, caused a dramatic shift in scientific interest and resources away from many enzymatic activities that were historically studied, such as ARAT, towards enzymatic activities that recognized CRBP-retinol as substrate. Despite subsequent studies which showed that in human skin, "free" retinol is esterified 2-fold more effectively than CRBP-bound retinol[138], doubt about the physiologic relevance of ARAT could not be shed. Because the K_m for "free" retinol as a substrate in the ARAT reaction is higher than that for the LRAT reaction[139,140], it was argued that in the presence of CRBP, intracellular concentrations of "free" retinol would be insufficient to activate the ARAT activity. Only after the generation of *Lrat*$^{-/-}$ mice and the discovery that the enzyme encoded by *Dgat1* possesses ARAT activity did scientific interest in ARAT again gain momentum.[51,141,142] The major questions that are of importance to address include determining whether the enzyme encoded by *Dgat1* functions as an ARAT *in vivo* and the role ARAT plays in the regulation of retinoid homeostasis.

Retinoid homeostasis. RA regulates the expression of a large number of genes by activating nuclear retinoid receptors and affects numerous signaling pathways through RXR heterdimerization with other nuclear receptor transcription factors including PPAR, LXR, TR, VDR and other orphan nuclear receptors. Although regulation of the enzymes that oxidize retinol, *i.e.* retinol and retinal dehydrogenases, is likely an important mechanism for maintaining retinoid homeostasis, relevant to skin[47,143,144], not much is known about these regulatory mechanisms. In fact, overall very little is understood about the mechanisms that maintain retinoid homeostasis in the skin and other retinoid target tissues.

Studies examining the effects of RA treatment on human epidermis and cultured human keratinocytes suggest that RA autoregulates its own biosynthesis. Exogenous RA increases CRBPI [89,145], CRABPII[125,146], and CYP26[111] mRNA expression levels. Increased expression of these proteins presumably act to enhance retinol esterification, to sensitize cells to RA and activate feedback signals to decrease endogenous RA synthesis, and to accelerate the degradation of RA, respectively. Whether RA regulates the expression of retinoic acid biosynthesis enzymes is unknown. In some cell types, retinoic acid biosynthesis enzymes were found to be induced during limiting retinoid availability, suggesting increased expression of retinoic acid biosynthesis genes is necessary for maintaining adequate levels of RA from low levels of retinol.[147] Whether downregulation of retinoic acid synthesis enzymes is involved in decreasing endogenous retinoic acid levels is unknown.

Studies in human epidermis and cultured human keratinocytes also suggest that the esterification of retinol with fatty acyl moieties may regulate RA biosynthesis by controlling substrate availability. Exogenous RA induced both ARAT and LRAT retinol esterification activities in cultured human keratinocytes and this induction increased the conversion of retinol to retinyl esters and reduced the conversion of retinol to retinoic acid by 50%.[138] It was postulated that RA induction of retinol esterification activity restricts endogenous RA biosynthesis. However, whether endogenous RA autoregulates its own biosynthesis and activity using similar mechanisms are unknown. Other studies have shown that topical ROL can exert the same effects as topical RA on the epidermis without eliciting the same degree of toxicity because the majority of the retinol is converted to retinyl esters without generating measurable levels of RA. [148,149] Because copious levels of retinol may drive RA biosynthesis, this suggests that a function of retinol esterification is to sequester excess

retinol that exceeds the RA signaling needs of the tissue. Taken together, these findings support a RA homeostatic function for retinol esterification, however, whether retinol esterification is physiologically required for the maintenance of RA homeostasis has not been established and the relative contributions of LRAT and ARAT activities to this function is currently unknown.

Our lab recently determined that the microsomal enzyme encoded by *Dgat1* also possesses ARAT activity in *in vitro* assays.[141] However, it is currently unclear whether DGAT1 functions as an ARAT enzyme *in vivo* and this question is addressed by the studies presented in this thesis.

Acyl CoA:diacylglycerol acyltransferase 1 (DGAT1)

DGAT enzymes and Dgat1 deficiency in mice. *Dgat1* was originally identified by our lab as a gene that encodes an acyl CoA:diacylglycerol acyltransferase (DGAT). The DGAT enzyme functions in triacylglycerol metabolism by joining diacylglycerol with a fatty acyl-CoA moiety to form triacylglycerol.[150,151] *Dgat1* is highly expressed in mammalian tissues that are important in triacylglycerol metabolism, including the small intestine, liver, mammary gland, and adipose tissue.[151] *Dgat1* is also highly expressed in the sebaceous glands of the skin [152].

Mice lacking *Dgat1* (*Dgat1*$^{-/-}$ mice) exhibit a surprisingly pleiotropic phenotype. They exhibit increased energy expenditure, resistance to diet-induced obesity, increased sensitivity to insulin and leptin, and defects in several epithelial tissues, including the mammary gland and skin.[153-158] Studies are ongoing in the lab to identify the mechanisms underlying this pleiotropic phenotype.

Dgat1 deficiency and skin defects. Initial characterization of the skin defects of *Dgat1*$^{-/-}$ mice revealed several abnormalities including abnormal sebum production by *Dgat1*$^{-/-}$ sebaceous glands, sebaceous gland atrophy, and alopecia, which is most prominent in male mice after puberty.[158] Analysis of hair lipids of *Dgat1*$^{-/-}$ mice revealed the absence of several lipids, including type II wax diesters, the most abundant component in murine hair lipids.[159] How *Dgat1* deficiency causes abnormal sebum secretion is currently unclear, however we recently found that the enzyme encoded by *Dgat1* also exhibits wax synthase activity *in vitro* suggesting loss of wax synthase activity in the sebaceous gland may be the cause of sebaceous gland dysfunction.[141]

Interestingly, when *Dgat1*$^{-/-}$ mice were crossed with leptin-deficient *ob/ob* mice, not only were the metabolic benefits of *Dgat1* deficiency eliminated, the skin and hair defects were also abolished.[158] When leptin replacement therapy was administered to the *ob/ob*;*Dgat1*$^{-/-}$ mice via intracerebroventricular (ICV) infusion to examine the contribution of central leptin signaling to the *Dgat1*$^{-/-}$ phenotype, ICV leptin caused sebaceous gland function to deteriorate.[158] Remarkably, upon the withdrawal of ICV leptin, sebaceous gland function began to recover.[158]

Although the sebaceous gland defects of *Dgat1*$^{-/-}$ mice have been well characterized, the exact nature of the alopecia of *Dgat1*$^{-/-}$ mice is still unclear. Unpublished studies conducted by Dr. Hubert Chen demonstrated that hair growth could be stimulated in the alopecic *Dgat1*$^{-/-}$ mice by topical treatment of PKC activators, such as the phorbol ester PMA, which are known to activate hair growth in wild-type mice. Unexpectedly hair growth was also induced in alopecic *Dgat1*$^{-/-}$ mice after two weeks of intraperitoneal injections of

pIpC (an inducer of interferons). Whether pIpC also stimulates hair growth in wild-type mice is unknown.

DGAT1 is a multifunctional acyltransferase. Recent findings in the lab suggest that the pleiotropic phenotype of *Dgat1*$^{-/-}$ mice may, in part, be due to additional acyltransferase activities of the enzyme encoded by *Dgat1*. Dr. Eric Yen found that besides diacylglycerol, the enzyme encoded by *Dgat1* recognizes other substrates including retinol, monoacylglycerol, and alcohols, and synthesizes retinyl esters, diacylglycerol, and waxes, respectively, in *in vitro* assays.[141] However, it is currently unclear whether these *in vitro* acyltransferase activities of DGAT1 are physiologically relevant and whether any of the phenotypes of *Dgat1* deficiency is related to these other acyltransferase activities of DGAT1. Interestingly, the skin phenotype of *Dgat1*$^{-/-}$ mice - adult-onset alopecia and sebaceous gland atrophy[158] - resemble well-established toxicity symptoms associated with systemic or topical retinoids[4,41,105,148], suggesting this aspect of the phenotype of *Dgat1*$^{-/-}$ mice may be related to the ARAT activity of DGAT1.

Outline of thesis

The goal of this thesis was to test the hypothesis that the enzyme encoded by *Dgat1* functions as an ARAT enzyme in the skin and plays a role in maintaining retinoid homeostasis in the skin. To test this hypothesis, we studied the alopecia phenotype of *Dgat1*$^{-/-}$ mice and determined whether altered retinoid metabolism in the skin of *Dgat1*$^{-/-}$ mice plays a role in the development of alopecia. We first conducted a systematic analysis of the alopecia phenotype of *Dgat1*$^{-/-}$ mice. We examined hair cycle progression during the first

two postnatal hair cycles of *Dgat1*$^{-/-}$ mice, determined the pattern of alopecia development, and elucidated the proximal cause of the alopecia in *Dgat1*$^{-/-}$ mice (Chapter 2). We then examined retinoid metabolism in *Dgat1*$^{-/-}$ skin by examining retinoid levels and retinoid activity in the skin of retinoid-replete and retinoid-deplete *Dgat1*$^{-/-}$ mice (Chapter 3). To directly assess the role of retinoids in the development of the alopecia, we employed dietary retinoid deprivation and observed the effects of retinoid depletion on the alopecia of *Dgat1*$^{-/-}$ mice (Chapter 3). To determine if DGAT1 plays a protective role against retinoid toxicity, we assessed the susceptibility of *Dgat1*$^{-/-}$ skin and mouse embryonic fibroblasts to retinol-induced toxicity (Chapter 3). Finally, we determined whether the alopecia of *Dgat1*$^{-/-}$ mice is caused by local or systemic affects of *Dgat1* deficiency by generating mice with selective deletion of *Dgat1* in the epidermis using the Cre/lox P recombination method (Chapter 3).

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CHAPTER II.

Dgat1 Modulates Hair Cycling in Mice

Dgat1 Modulates Hair Cycling in Mice

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Summary

Alopecia, whether caused by hair disorders or by the treatment of cancer with chemo- and radiation therapy, can have a significant psychological impact on the individuals who suffer from it. Studies in animal models of alopecia further our understanding of hair growth and enable us to develop more effective pharmacological tools to treat alopecia. Mice lacking the acyl CoA:diacylglycerol acyltransferase 1 (DGAT1) enzyme develop adult-onset alopecia, the nature of which is unclear. Here we show that *Dgat1*$^{-/-}$ mice exhibit cyclical alopecia due to excessive hair shedding accompanied by alterations in hair cycling including a prolonged first anagen phase and the precocious onset of the second anagen phase at ~8

weeks of age. The excessive shedding of the first anagen club hairs is temporally linked to the precocious onset of second anagen. *Dgat1* exhibits cycle-dependent expression with highest mRNA levels in the telogen phase suggesting a role for *Dgat1* in maintaining hair follicles in telogen. We conclude that the alopecia of *Dgat1*$^{-/-}$ mice results from effect of *Dgat1*-deficiency on the mechanisms of hair shedding and not hair cycle arrest.

Introduction

Discoveries in hair biology have contributed to not only our basic understanding of hair growth, but also to developmental biology, stem cell biology, and diseases, such as obesity (*e.g.*, agouti proteins) and cancer (*e.g,* patched proteins). However, one of the driving forces behind hair research is its profound clinical relevance. Alopecia, whether caused by hair disorders or by the treatment of cancer with chemo- and radiation therapy, is a common problem in both men and women and can have a significant psychological impact on the individuals who suffer from it.

Hair is produced by the hair follicle, a complex mini-organ that undergoes periodic cycles of growth and involution. During each cycle, the new hairs produced replace old club hairs of the previous cycle. During hair follicle cycling, dramatic morphological transformations occur in the hair follicle as it transitions from a resting (telogen) phase to a growth (anagen) phase, with rapid proliferation of follicular keratinocytes and elongation and thickening of the hair shaft, to an apoptosis driven regression (catagen) phase.[1] Furthermore, the hair follicle environment including the extracellular matrix, the perifollicular vasculature, the immune environment, melanocytes, and perifollicular innervation also undergo dramatic coordinated cyclical changes in proliferation, differentiation, and apoptosis.[2] Although the precise regulatory mechanisms that drive hair follicle cycling are still unclear, studies of animal models of alopecia have furthered our understanding of how hair grows and cycles. Mice are an excellent model for identifying the effects of genetic and pharmaceutical manipulations on hair growth because subtle changes in the hair cycle and alterations in the morphology of hair follicles can be easily detected using histological methods because the progression of dorsal skin hair follicles through the first two postnatal hair cycles follows a

predictable time-scale and occurs synchronously. Studies in mice have established that the process of hair cycling depends on the interaction between the epithelial and mesenchymal components of the skin and is modulated by various hormones, cytokines, neuropeptides, neurotransmitters, and neurotrophins. [2] Also causes of alopecia in people and mice include undesired alterations in hair follicle cycling (i.e. shortened anagen or telogen arrest), defects in hair follicle regeneration (i.e. inflammation and autoimmune destruction of the stem cell population of the skin)[3], and hair retention (i.e. increased hair shedding).[3-6] Continued investigation of new mouse models of alopecia is necessary for identifying new players involved in the regulation of hair cycling and reveal new targets for developing more effective pharmacological tools to treat alopecia.

One animal model of alopecia that has yet to be investigated is mice lacking acyl CoA:diacylglycerol acyltransferase 1 (*Dgat1*$^{-/-}$ mice).[7] DGAT1 was originally identified as one of two known enzymes that catalyze the last step in triacylglycerol synthesis by esterifying diacylglycerol with a fatty acyl-CoA molecule.[8] DGAT1 is highly expressed in all mammalian tissues that are important in triacylglycerol metabolism, including the small intestine, liver, mammary gland, and adipose tissue.[8] DGAT1 is also highly expressed in the sebaceous glands of the skin [9]. Mice lacking DGAT1 (*Dgat1*$^{-/-}$ mice) exhibit a surprisingly pleiotropic phenotype. In addition to being resistant to diet-induced obesity [10], these mice also exhibit abnormalities in skin and fur function.[11] Initial studies conducted on the skin and fur function of *Dgat1*$^{-/-}$ mice revealed several abnormalities including abnormal sebum production by *Dgat1*$^{-/-}$ sebaceous glands, sebaceous gland atrophy, and alopecia, which is most prominent in male mice after puberty.[11] Although the sebaceous gland defects of *Dgat1*$^{-/-}$ mice have been well characterized, the exact nature of the alopecia of *Dgat1*$^{-/-}$ mice

has not been determined. In this study, I conduct a systematic analysis of the alopecia phenotype of *Dgat1*$^{-/-}$ mice.

Results

Altered hair cycling in *Dgat1*$^{-/-}$ mice

Histological analysis revealed that hair cycling progressed similarly in wild-type and *Dgat1*$^{-/-}$ follicles through morphogenesis until the transition from the first telogen phase to the first anagen phase (Fig.1a).

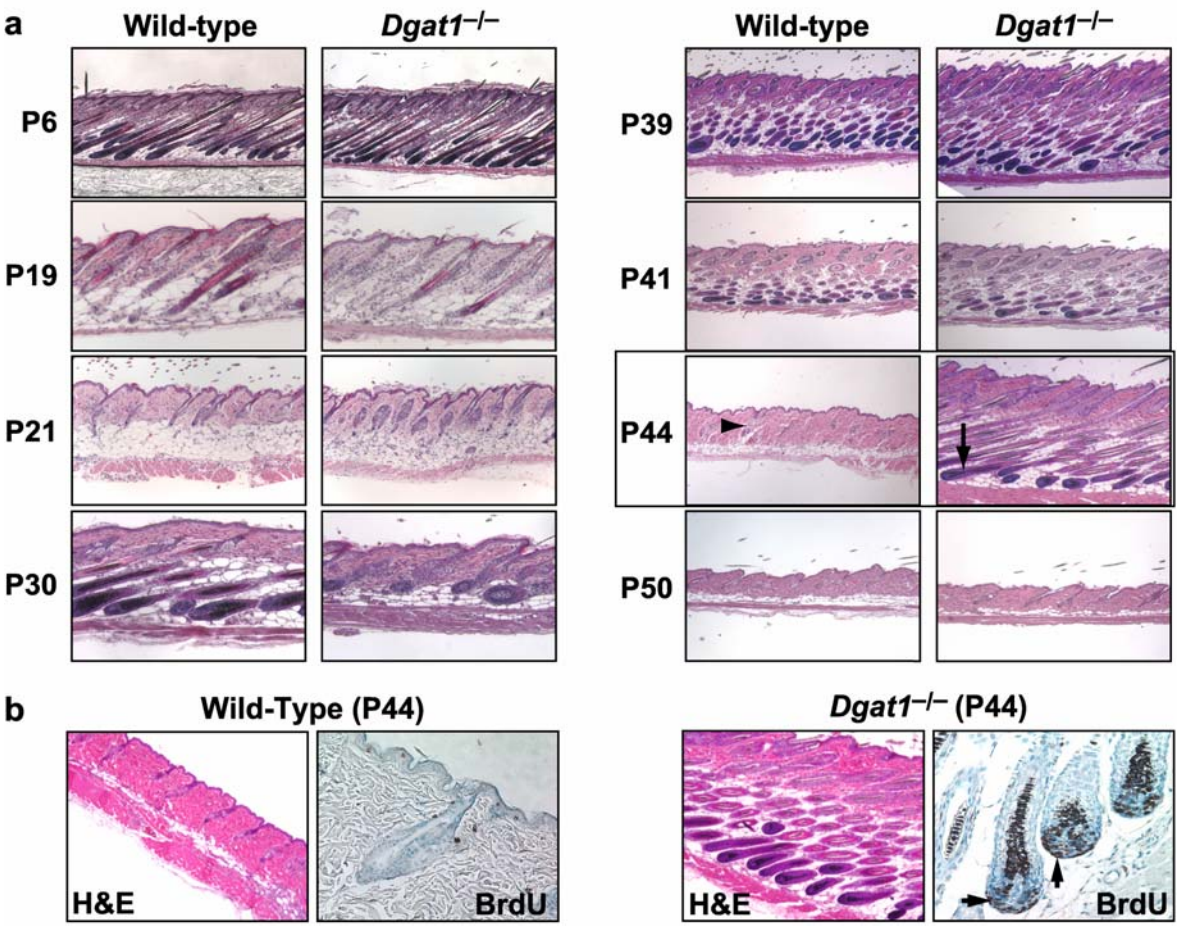

Figure 1. Prolonged first anagen phase in *Dgat1*$^{-/-}$ mice. (a) Skin sections were stained with haematoxylin and eosin. Hair follicles of P44 wild-type mice were in telogen (arrowhead), whereas hair follicles of P44 *Dgat1*$^{-/-}$ mice were in anagen (arrow) (n = 6 per genotype for P44, n = 3 per genotype all other time-points). (b) Presence of proliferating matrix keratinocytes in hair follicles of P44 *Dgat1*$^{-/-}$ mice. BrdU-positive matrix keratinocytes (arrow) (n = 6 per genotype).

The appearance of P39 and P41 (late first-anagen phase) follicles in wild-type and *Dgat1*$^{-/-}$ skin was also similar (Fig. 1a). However, at P44 the *Dgat1*$^{-/-}$ follicles were in anagen (extending into the intradermal fat layer) rather than telogen phase, suggesting that the first anagen phase was prolonged (Fig. 1a). This was confirmed by increased BrdU staining in the bulbs of P44 *Dgat1*$^{-/-}$ follicles (Fig. 1b), indicating active proliferation of hair matrix keratinocytes, a characteristic of anagen phase. At P50, *Dgat1*$^{-/-}$ follicles had entered telogen phase, like those of wild-type mice (Fig. 1a).

In addition to having a prolonged first anagen phase, *Dgat1*$^{-/-}$ follicles also exhibited precocious entry into second anagen phase. Whereas wild-type mice remained in telogen

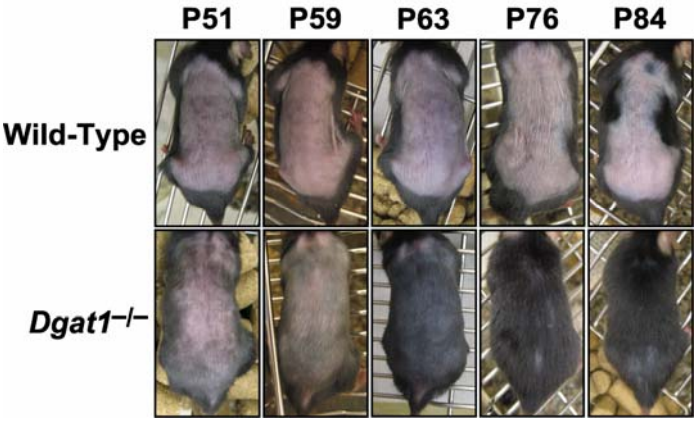

Figure 2. Precocious onset of second anagen in *Dgat1*$^{-/-}$ mice. *Dgat1*$^{-/-}$ and wild-type mice were shaved on postnatal (P) day 51 and photographed successively at various time-points. Precocious anagen onset is evident in *Dgat1*$^{-/-}$ mice by appearance of new hair at P63 and full hair coat by P76 (*n* = 5 per genotype for P63).

Figure 3. Schematic summary of the first two postnatal hair cycles of *Dgat1*$^{-/-}$ mice compared with wild-type mice. A, anagen (black bar); C, approximated period of catagen (gray bar); and T, telogen (white bar).

phase from P51 to P76, evidenced by lack of hair growth in shaved mice, *Dgat1*$^{-/-}$ mice exhibited hair growth by P63 (Figure 2). The alterations in the first two hair cycles of *Dgat1*$^{-/-}$ mice are schematically shown in Figure 3.

Hair cycle dependent expression pattern of *Dgat1* in whole skin

The alterations in the hair cycle of *Dgat1*$^{-/-}$ mice suggested a phenotypic relationship between *Dgat1* expression and hair cycling, we examined *Dgat1* mRNA expression in whole skin of wild-type mice during a depilation-induced hair cycle. The highest levels of *Dgat1* mRNA were detected during the telogen phase, suggesting a role for DGAT1 in maintaining the hair follicle in quiescence (Fig. 4).

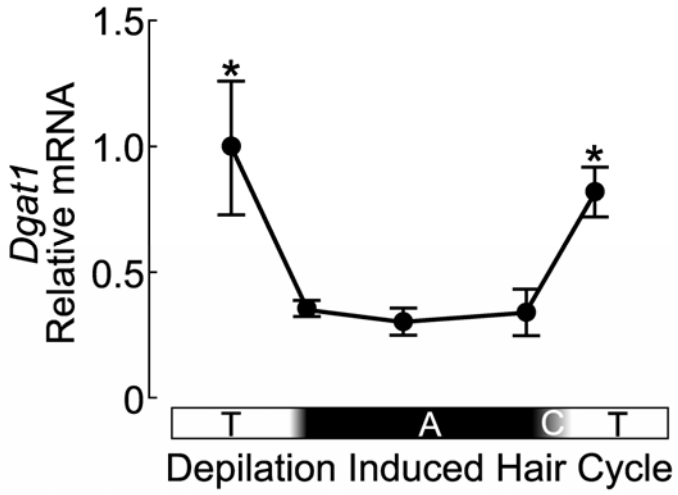

Figure 4. Hair cycle dependent mRNA expression of *Dgat1*. Wild-type mice were depilated on P50 and skin harvested on days 0, 1, 8, 17, and 22 post-depilation. *Dgat1* mRNA levels in the whole skin were quantified by real-time PCR. (n = 4–5 mice per time-point). *$P < 0.05$ *versus* non-telogen time-points.

***Dgat1*$^{-/-}$ mice exhibit cyclical alopecia**

We next examined the pattern of alopecia development in *Dgat1*$^{-/-}$ mice. Alopecia generally first appeared as a prominent bald patch in the dorsal caudal region of *Dgat1*$^{-/-}$

mice around 9 weeks of age, coincident with the precocious onset of second anagen phase (Fig. 5a, white box). The skin within the bald patch was gray, and new hairs emerged from the skin a few days later. By 11 weeks of age, the hair had re-grown (Fig. 5a, white box). Generally the restored hair growth was temporary. Another cycle of patchy alopecia in a different location usually ensued followed by hair re-growth during the following weeks (data not shown). This patchy cyclical alopecia persisted in older (5–7 months-old) *Dgat1*$^{-/-}$ mice (Fig. 5b, boxes indicate development of bald patches followed by re-growth). Older *Dgat1*$^{-/-}$ mice also exhibited generalized hair thinning (Fig. 5b), most likely due to an age-related decline in the rate of hair growth combined with excessive hair loss. The cyclical nature of the alopecia and the increased propensity of *Dgat1*$^{-/-}$ hair follicles to be in anagen suggested that hair loss, rather than telogen arrest, was the primary cause of alopecia.

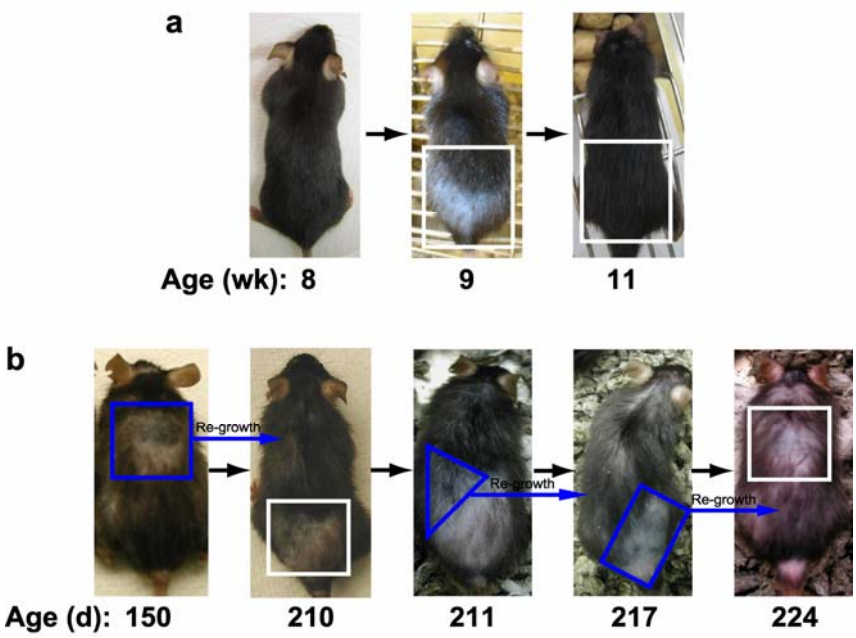

Figure 5. Cyclical alopecia in *Dgat1*$^{-/-}$ mice. (a) *Dgat1*$^{-/-}$ mouse photographed successively at 8, 9, and 11 weeks of age. Note the first appearance of alopecia (white box) in the dorsal caudal skin region at 9 weeks of age and complete hair re-growth in the same skin region (white box) by 11 weeks of age. (b) *Dgat1*$^{-/-}$ mouse photographed successively from 5 to 7.5 months of age. Note hair re-growth in alopecic areas and development of new alopecic areas (white boxes) at different locations.

Excessive hair shedding of *Dgat1*$^{-/-}$ mice is temporally linked to the precocious onset of second anagen

The coincident appearance of alopecia with the aberrant onset of the second anagen phase in *Dgat1*$^{-/-}$ mice suggested that the premature shedding of the first anagen club hairs may be responsible for the precocious onset of the second anagen phase. We therefore utilized an adhesive tape hair-loss test[6] to determine when hair becomes prone to shedding. Surprisingly, at P52, when the hair cycles of wild-type and *Dgat1*$^{-/-}$ mice were in telogen, similar amounts of hair were removed by tape (Figs. 6a-b). Only after the onset of second anagen (P63) were significantly more hairs removed by tape from *Dgat1*$^{-/-}$ mice (Figs. 6a-b).

Consistent with the results of the adhesive tape hair loss test, *Dgat1*$^{-/-}$ mice do not exhibit alopecia until after the precocious onset of the second anagen phase (Fig. 6c). Because telogen clubs hairs are naturally shed during exogen, a hair cycle phase linked to anagen[12], we applied the adhesive tape hair loss test to P28 hair, when the hair cycles of wild-type and *Dgat1*$^{-/-}$ mice were synchronized in anagen. We found that similar amounts of hair was removed by tape from wild-type and *Dgat1*$^{-/-}$ mice and the amounts were significantly less compared with the amount removed from *Dgat1*$^{-/-}$ mice at P63 (Fig. 6a-b). We also examined all hairs removed by tape by microscopy revealed that the club ends were intact (not shown), confirming that premature shedding rather than breakage was the cause of hair loss at P63.

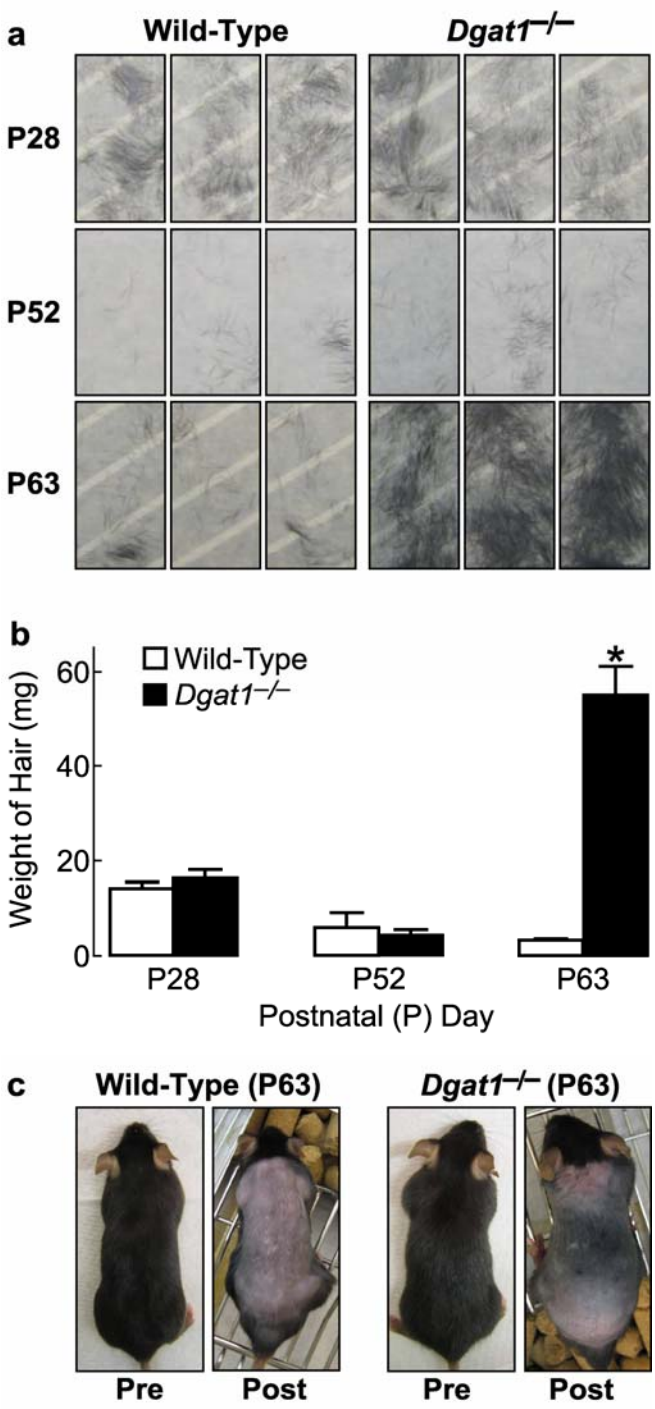

Figure 6. Excessive hair shedding in *Dgat1*$^{-/-}$ mice. (a) Adhesive tape hair loss test performed on dorsal hair of wild-type and *Dgat1*$^{-/-}$ mice (n = 3 per genotype, each tape represents one individual mouse). (b) Quantification of hair removed by adhesive tape (n = 3 per genotype). *P < 0.001 *versus* wild type. (c) The precocious onset of second anagen takes places prior to the appearance of alopecia. *Dgat1*$^{-/-}$ mice that were shaved at P63, prior to the appearance of alopecia, had graying skin indicative of anagen initiation whereas skin of wild-type mice was pink indicative of telogen (n = 5 per genotype).

Discussion

The precise regulatory mechanisms that drive hair follicle cycling are still unclear, but studies of animal models of alopecia have furthered our understanding of how hair grows and cycles. The goal of our study was to further characterize the nature of alopecia of *Dgat1*$^{-/-}$ mice. Our analysis showed that hair shedding, not hair cycle arrest, is the proximal cause of alopecia of *Dgat1*$^{-/-}$ mice. *Dgat1*$^{-/-}$ mice exhibit a pattern of patchy cyclical alopecia initiated by the premature shedding of the first anagen club hairs between 8-9 weeks of age, which was immediately followed by hair re-growth. *Dgat1*$^{-/-}$ hair follicles also exhibit alterations in the hair cycle characterized by the increased propensity to be in anagen. Lack of overt signs of inflammation in the skin of *Dgat1*$^{-/-}$ mice (data not shown) and the presence of normal hair follicular morphology indicate that hair follicle degeneration or destruction is not involved in the development of alopecia in *Dgat1*$^{-/-}$ mice.

Defective hair anchorage caused by altered terminal differentiation of follicular and hair shaft keratinocytes is unlikely to be the cause of excessive hair shedding in *Dgat1*$^{-/-}$ mice. The first anagen club hairs were tightly anchored throughout the telogen phase (P52) and only became prone to shedding after the aberrant onset of second anagen at P63. The excessive shedding of the first anagen club hairs may be triggered by premature hyperactivation of mechanisms of exogen, a process of regulated hair shedding that normally takes place throughout the anagen phase to make room for the growth of new hairs.[12] Whether the precocious onset of second anagen plays a role in triggering excessive hair shedding by *Dgat1*$^{-/-}$ mice is currently unknown.

Our study of the alopecia phenotype of *Dgat1*$^{-/-}$ mice has revealed *Dgat1* as novel player involved in the regulation of hair cycling. We show that *Dgat1* expression in the skin

exhibited a hair cycle dependent pattern and was highest during the telogen phase. We also showed that loss of *Dgat1* resulted in the increased propensity of hair follicles to be in anagen. Taken together, these findings suggest a role for DGAT1 in the maintaining hair follicles in the quiescent telogen phase. DGAT1 may be a relevant pharmaceutical target treating certain types of alopecia.

Methods

Mice and diets. Male *Dgat1*$^{-/-}$ mice and wild-type mice were of C57BL/6J genetic background and genotyped as previously described.[7] Mice were housed in a pathogen-free barrier facility (12-h light/12-h dark cycle) and fed a standard chow (5053 PicoLab Diet; Purina, St. Louis, MO).

Histological analyses. Whole mid-dorsum skin was removed from wild-type and *Dgat1*$^{-/-}$ mice, fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) at 4°C overnight, and then washed for 15 min in PBS and embedded in paraffin. Tissue sections (6 μm) were stained with hematoxylin and eosin.

***In vivo* epidermal proliferation assay.** Bromodeoxyuridine (BrdU) labeling reagent (10 μl/g body weight; Invitrogen, Carlsbad, CA) was administered to mice (6 week old; 6 per each genotype) intraperitoneally two hr before skin harvesting. Paraffin-embedded tissue sections were prepared as described above. BrdU was detected using the BrdU staining kit (Invitrogen, Carlsbad, CA).

Real-time PCR. Whole skin was homogenized and total RNA extracted with RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA) RNA (5 μg) was reverse-transcribed using the Superscript III™ First-Strand Synthesis Supermix kit (Invitrogen, Carlsbad, CA). Real-time PCR was performed and analyzed with the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Each 20-μl PCR reaction contained 2 μl of cDNA, 10 μl of 2x SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) and10 pmol each of forward and reverse primer. Relative expression levels were calculated by the comparative C_T (cycle of threshold detection) method as outlined in the manufacturer's technical bulletin. Cyclophilin expression was used as control. The primers used for RT-PCR are: *Dgat1* sense 5′-TTCCGCCTCTGGGCATT-3′ and antisense 5′-AGAATCGGCCCACAATCCA-3′; *Cyclophilin* sense 5′-TGGAAGAGCACCAAGACAGACA-3′ and antisense 5′-TGCCGGAGTCGACAATGAT-3′.

Adhesive tape hair loss test. A 1 inch x 1.5 inch piece of adhesive tape (VWR, Batavia, IL) was pressed gently on the nape of the neck of mice and pulled off in the direction of hair growth (*i.e.*, toward the tail). Tape was photographed and weighed (before and after stripping).

Depilation to induce anagen. P50 mice were anaesthetized and dorsal hairs were depilated using sugar wax (Nair Microwave Wax, Church & Dwight Co., Princeton, NJ). Afterwards, skin biopsies were taken at various days for RNA extraction and histological analysis.

Statistical analyses. Values are reported as mean ± *s.e.m*. Means were compared by ANOVA followed by a Tukey test.

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CHAPTER III.

Dgat1 Is Essential for Retinoid Homeostasis in the Skin

Dgat1 Is Essential for Retinoid Homeostasis in the Skin

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Summary

Although retinoids are key regulators of skin and hair biology, the factors that regulate retinoid homeostasis in the skin are incompletely understood. We recently discovered that the microsomal enzyme encoded by *Dgat1,* previously shown to be important in triglyceride and energy metabolism, also possesses acyl CoA:retinol acyltransferase (ARAT) activity. Whether this enzyme functions as an ARAT *in vivo* is unknown. Here we show that *Dgat1* is required for maintaining retinoid homeostasis in the skin. *Dgat1*-deficient (*Dgat1*$^{-/-}$) mice had reduced retinol esterification, increased levels of retinol and retinoic acids, and increased

retinoic acid activity in the skin. Toxic effects associated with excess retinoic acids in the skin is the cause of the cyclical alopecia of *Dgat1*$^{-/-}$ mice because retinol deprivation in these mice reduced retinoic acids in the skin and prevented alopecia. *Dgat1*$^{-/-}$ skin was also more susceptible to topical retinol-induced toxicity. Selective deletion of *Dgat1* in the epidermis was sufficient to cause alopecia, indicating that the retinol acyltransferase functions of DGAT1 are epidermis-autonomous. We conclude that DGAT1 functions as a retinol acyltransferase in the epidermis, where it helps to maintain retinoid homeostasis, in part by preventing retinoid toxicity.

Introduction

Retinoids (retinol, or vitamin A, and its derivatives) play key roles in skin and hair biology.[1,2] Deficiency of retinoids results in thinning of the epidermis and atrophy of hair follicles and sebaceous glands.[1] On the other hand, excess retinoids induce epidermal hyperplasia, suppress keratinization, inhibit sebocyte proliferation and differentiation, impair barrier function, and alter hair growth.[1,3-5] The pleiotropic effects of retinoids make them efficacious pharmaceuticals for the treatment of dermatological diseases, including cystic acne, psoriasis, and other keratinization disorders.[4,6-8] However, the complex effects of retinoids also underscore the importance of retinoid homeostasis in normal skin biology.

Retinol exerts biological effects after metabolic activation into retinoic acid (RA)[9]. RA acts as a ligand for RA receptors (RAR)[10], which regulate many genes involved in cellular proliferation and differentiation[11]. Regulation of the enzymes that oxidize retinol, *i.e.* retinol and retinal dehydrogenases, is therefore an important mechanism for maintaining retinoid homeostasis, relevant to skin[12-14]. Esterification of retinol with fatty acyl moieties to form retinyl esters also contributes to skin retionid homeostasis[15]. Retinol esterification provides a means to maintain intracellular stores of retinol and to prevent toxicity from excessive levels of unesterified retinol, which can drive the biosynthesis of retinoic acids.

Two major retinol esterification activities exist. One is mediated by lecithin:retinol acyltransferase (LRAT), which catalyzes the covalent conjugation of a fatty acyl moiety from lecithin (phosphotidylcholine) with retinol bound to cellular retinol-binding proteins (CRBP).[16] LRAT plays a key role in maintaining tissue retinol stores, as demonstrated by studies of LRAT-deficient (*Lrat*$^{-/-}$) mice.[17-19] These mice have severe reductions in hepatic and lung retinyl ester levels[17-19], exhibit testicular hypoplasia/atrophy,[18] and develop

blindness[19], but apparently do no exhibit a skin phenotype. Levels of retinyl esters are normal in the white adipose tissue (WAT) and other tissues of LRAT-deficient mice, indicating the existence of alternative mechanisms for retinol esterification.[17,18] This esterification is likely mediated by acyl CoA:retinol acyltransferase (ARAT), which uses fatty acyl CoA as co-substrate and functions only with "free" retinol, *i.e.* retinol not bound with CRBP.[16,20] ARAT activity is found in many tissues, including skin, but prior to production of *Lrat*$^{-/-}$ mice, was considered a minor participant in retinol esterification in tissues that express CRBP.[20-23]

A role for retinol esterification in retinoid homeostasis in the skin has not yet been established, nor has the relative contribution of the LRAT and ARAT activities to this role because, until recently, a gene encoding an ARAT was unknown. However, we and others showed that the microsomal enzyme encoded by *Dgat1*, an acyl CoA:diacylglycerol acyltransferase (DGAT) that catalyzes the last step in triacylglycerol synthesis[24], also possesses ARAT activity in *in vitro* assays.[25,26] Furthermore, several tissues of *Dgat1*-deficient (*Dgat1*$^{-/-}$) mice had marked reductions in ARAT activity, and murine embryonic fibroblasts (MEFs) lacking *Dgat1* had reduced retinol esterification.[25] *Dgat1*$^{-/-}$ mice exhibit a pleiotropic phenotype that includes resistance to diet-induced obesity, increased sensitivity to insulin and leptin, and defects in several epithelial tissues, including the mammary gland and skin.[27-32] Interestingly, the skin manifestations of *Dgat1*$^{-/-}$ mice, which include alopecia and sebaceous gland atrophy[32], resemble those of retinoid toxicity[4,33], suggesting this aspect of the phenotype of *Dgat1*$^{-/-}$ mice may be related to the ARAT activity of DGAT1.

In chapter 2, we carried out a detailed analysis of the alopecia phenotype of *Dgat1*$^{-/-}$ mice and identified excessive hair shedding as the cause of cyclical alopecia. In this study, we tested the hypothesis that DGAT1 functions as an ARAT in the skin, where it serves to

maintain retinoid homeostasis and prevent retinol toxicity. We determined if retinoid metabolism was altered in *Dgat1*$^{-/-}$ skin and characterized the alopecia of *Dgat1*$^{-/-}$ mice, determining whether the effects of dietary retinol deprivation affected this aspect of the phenotype. We also directly assessed the susceptibility of *Dgat1*$^{-/-}$ skin to retinol toxicity. Finally, we assessed whether the retinoid metabolism aspects of *Dgat1* function were tissue-autonomous by deleting the gene specifically in the epidermis.

Results

Altered retinoid metabolism in *Dgat1*$^{-/-}$ skin

To determine if DGAT1 contributes to retinol esterification in skin, we measured ARAT activity in whole skin homogenates of 7-week-old wild-type and *Dgat1*$^{-/-}$ mice fed a chow diet. ARAT activity was reduced by ~90% in *Dgat1*$^{-/-}$ skin (Fig. 1). As expected, DGAT activity was reduced by more than 95%. The activity of a control enzyme, acyl

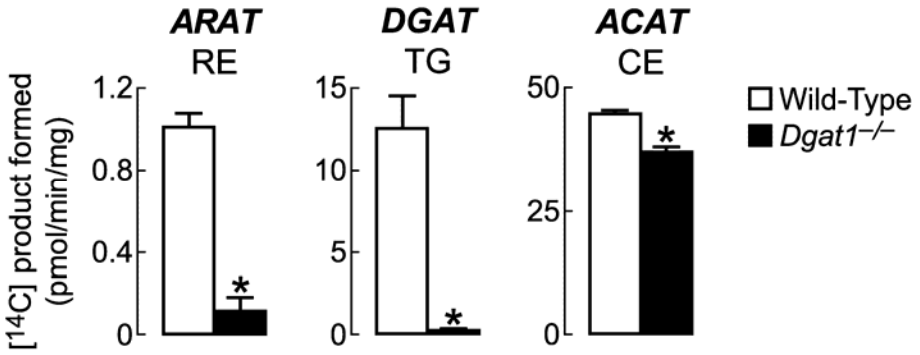

Figure 1. Reduced *in vitro* ARAT activity in whole skin of *Dgat1*$^{-/-}$ mice (age 7 weeks, $n = 7$ per genotype). *****$P < 0.001$ *versus* wild type.

CoA:cholesterol acyltransferase (ACAT), which catalyzes cholesterol ester synthesis, was slightly lower in *Dgat1*$^{-/-}$ skin, most likely due to the atrophy of the sebaceous glands where ACAT enzymes are highly expressed[34] (Fig. 1).

The decreased ARAT activity suggested that retinoid homeostasis might be altered in *Dgat1*$^{-/-}$ skin. To assess this, we measured levels of retinoids in whole skin of *Dgat1*$^{-/-}$ and wild-type mice that were fed a formulated purified diet containing the AIN recommended amount of retinol, *i.e.* a retinol-sufficient (RS) diet. Interestingly, retinyl ester levels were similar in *Dgat1*$^{-/-}$ and wild-type skin (Fig. 2a). However, levels of retinol and all-*trans*-retinoic acid (all-*trans*-RA) were increased ~22% and 40%, respectively, in *Dgat1*$^{-/-}$ skin

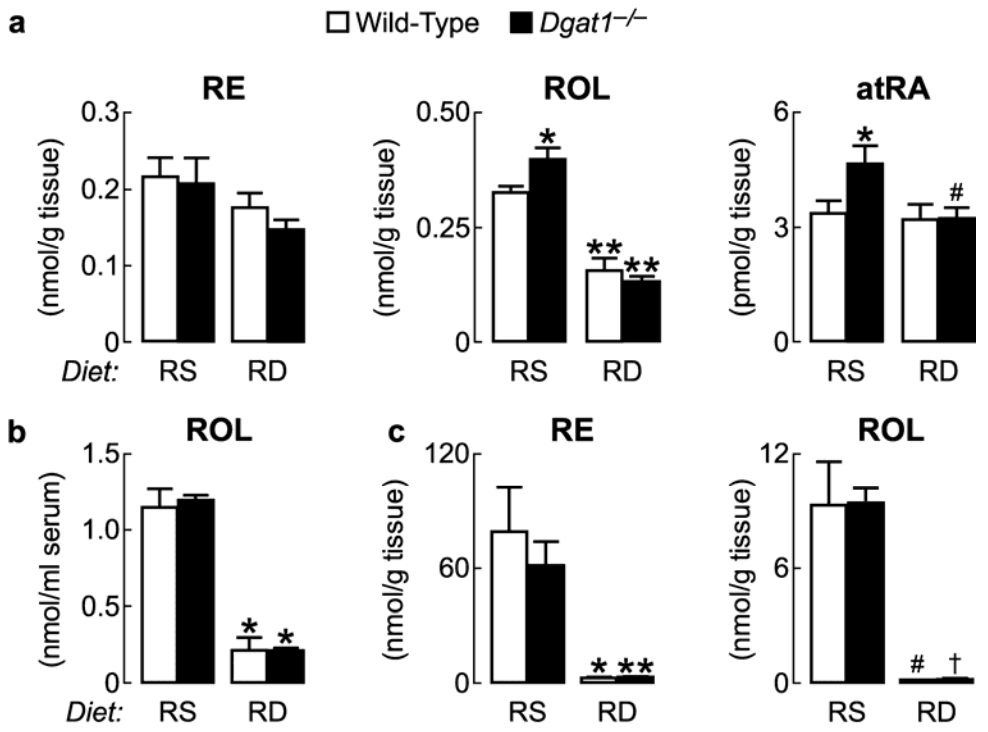

Figure 2. Altered retinoid homeostasis in *Dgat1*$^{-/-}$ skin. (a) Increased retinol (ROL) and all-*trans*-retinoic acid (atRA) concentrations in whole skin of retinoid-sufficient (RS) but not retinoid-deficient (RD) *Dgat1*$^{-/-}$ mice (ages 7.5–14 weeks, $n = 4$–6 per genotype). *$P < 0.05$ *versus* wild type; **$P < 0.001$, # $P < 0.05$ *versus* RS diet. (b) Similar serum ROL concentrations in wild-type and *Dgat1*$^{-/-}$ mice (ages 7.5–14 weeks, $n = 4$–6 per genotype). *$P < 0.001$ *versus* RS diet. (c) Similar hepatic RE and ROL concentrations in wild-type and *Dgat1*$^{-/-}$ mice (ages 7.5-14 weeks, $n = 4$–6 per genotype). *$P < 0.05$, **$P < 0.01$, $^{\#}P = 0.01$, and $^{\dagger}P < 0.001$ *versus* RS diet.

(Fig. 2a). Levels of serum retinol (Fig. 2b) and liver retinoids (Fig. 2c) were similar in *Dgat1*$^{-/-}$ and wild-type mice, suggesting that the increase in the all-*trans*-RA levels of *Dgat1*$^{-/-}$ skin most likely resulted from local imbalances in retinoid metabolism rather than differences in systemic retinol stores.

Because all-*trans*-RA mediates the majority of retinol's biological activities through transcriptional mechanisms[35], we examined mRNA levels of two all-*trans*-RA target genes—cellular retinol-binding protein I (*CrbpI*)[11,35] and cellular retinoic acid–binding protein II (*CrabpII*)[11,35]—in *Dgat1*$^{-/-}$ skin of chow-fed mice. The levels of these mRNAs were increased by 7- and 2-fold, respectively, in 7 week-old *Dgat1*$^{-/-}$ skin (Fig. 3). In contrast, the mRNA levels of *β-catenin* and vascular endothelial growth factor (*Vegf*), which are not targets of all-*trans*-RA, were similar in *Dgat1*$^{-/-}$ and wild-type skin (Fig. 3).

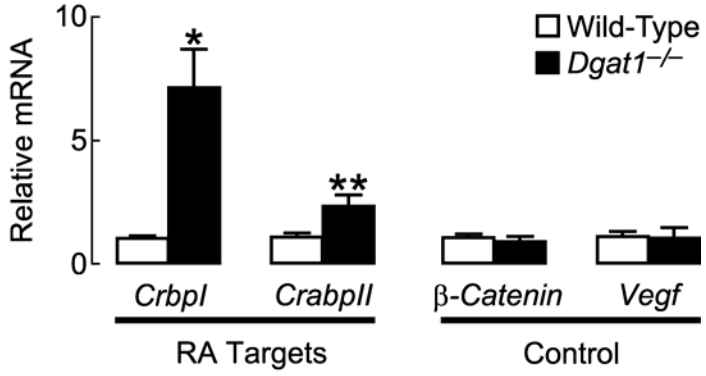

Figure 3. Increased expression of RA target genes in the whole skin of *Dgat1*$^{-/-}$ mice fed the retinoid-abundant chow diet. mRNA levels were quantified by real-time PCR (age 7 weeks, $n = 5–6$ per genotype). $^{*}P < 0.01$ and $^{**}P < 0.05$ *versus* wild type.

Retinoid deprivation prevents alopecia of *Dgat1*$^{-/-}$ mice

In Chapter 2, our analysis of the alopecia phenotype of *Dgat1*$^{-/-}$ mice revealed that they exhibit cyclical alopecia due to excessive hair shedding accompanied by alterations in hair cycling including a prolonged first anagen phase and the precocious onset of the second

anagen phase at ~8 weeks of age. We hypothesized that reducing retinoid levels in the skin may prevent excessive hair shedding and alopecia in *Dgat1*$^{-/-}$ mice. To test this hypothesis, we employed dietary retinoid deprivation to deplete retinoids from the skin. Both wild-type and *Dgat1*$^{-/-}$ mice on a retinoid-deficient (RD) diet had markedly decreased levels of serum retinol and hepatic retinoids (Figs. 2b-c). Although skin retinyl esters were not significantly reduced in mice of either genotype, the retinol content was similarly reduced by more than 50% in skin of wild-type and *Dgat1*$^{-/-}$ mice (Fig. 2a). Further, the levels of all-*trans*-RA were not elevated in skin of *Dgat1*$^{-/-}$ mice fed this RD diet (Fig. 2a).

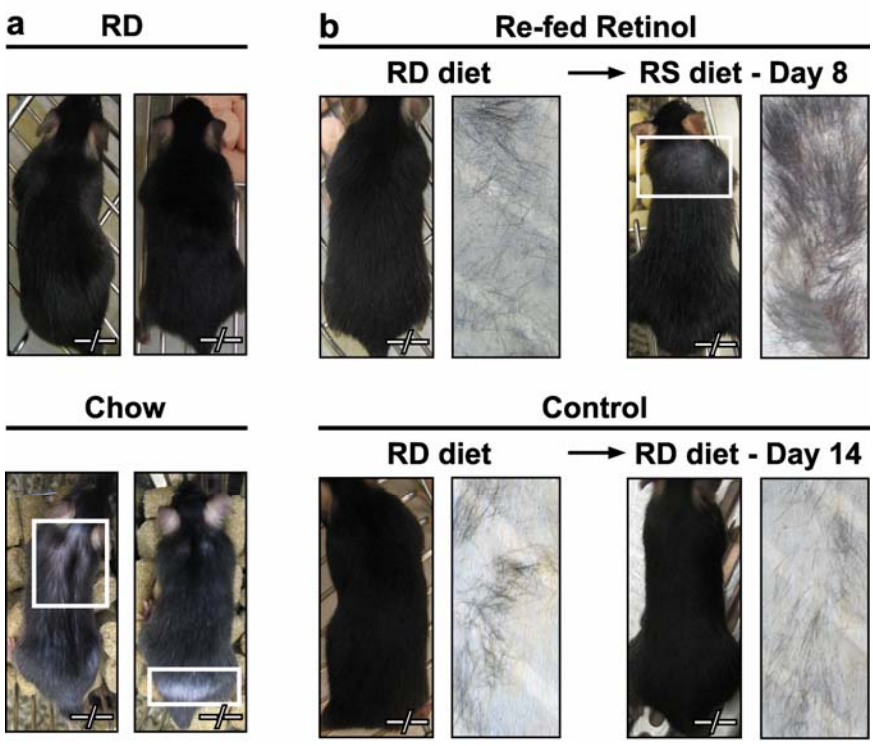

Figure 4. Retinoid deprivation prevents the development of alopecia in *Dgat1*$^{-/-}$ mice. (a) Retinoid depleted *Dgat1*$^{-/-}$ mice did not develop alopecia by 13 weeks of age whereas chow fed *Dgat1*$^{-/-}$ mice have developed prominent alopecia by 13 weeks of age. (n = 6 for retinoid depleted *Dgat1*$^{-/-}$ mice). (b) Re-feeding retinoid depleted *Dgat1*$^{-/-}$ mice the RS diet induced alopecia in as quickly as 8 days. (RS diet started at age 13 weeks for re-fed retinol group and control group was observed from age 13 to15 weeks, n = 3 per group).

On the RD diet, *Dgat1*$^{-/-}$ mice did not exhibit alopecia, whereas control chow-fed *Dgat1*$^{-/-}$ mice did after 9 weeks of age as expected (Fig. 4a). Furthermore, when retinoid-depleted *Dgat1*$^{-/-}$ mice were re-fed a RS diet, they developed alopecia after a week and exhibited excessive hair loss with an adhesive tape test (Fig. 4b). In contrast, *Dgat1*$^{-/-}$ mice maintained on the RD diet did not exhibit excessive hair loss (Fig. 4b).

The lack of alopecia in *Dgat1*$^{-/-}$ mice on a RD diet was accompanied by reduced retinoid activity in the skin. Although the RD diet did not completely normalize the mRNA levels of *CrbpI* and *CrabpII* in *Dgat1*$^{-/-}$ skin, levels were reduced by ~44% and 52%, respectively (Fig. 5). In *Dgat1*$^{-/-}$ mice, retinoid depletion also delayed the early onset of the second anagen phase by 5–12 days (Fig. 6).

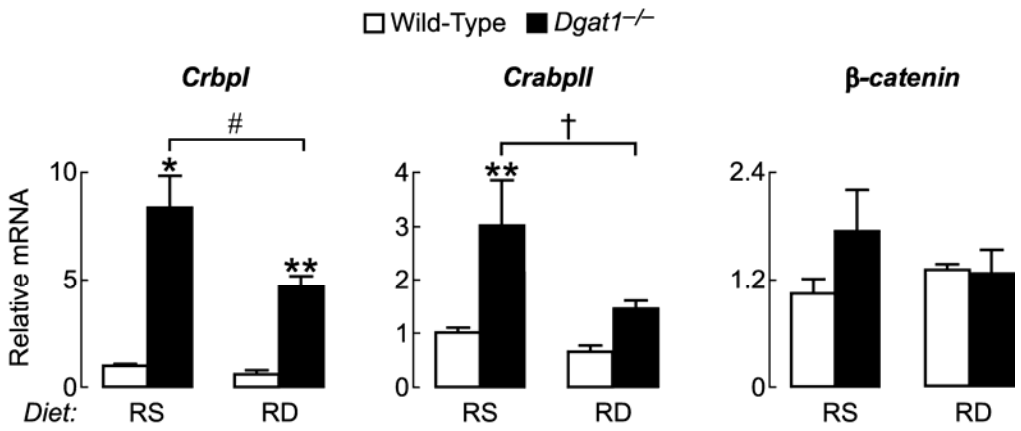

Figure 5. mRNA levels of RA target genes in retinoid-depleted *Dgat1*$^{-/-}$ mice are decreased compared with *Dgat1*$^{-/-}$ mice fed the retinoid sufficient diet. (age 7.5-14 weeks, $n = 2–6$ mice per group). ***$P < 0.001$ and **$P < 0.05$ *versus* wild type and # $P < 0.05$ and †trends lower $P = 0.07$ *versus* RS diet. mRNA levels of *CrabpII* in skin of RD diet fed wild-type and *Dgat1*$^{-/-}$ mice were not compared statistically because of insufficient skin samples from the RD diet fed wild-type mice.

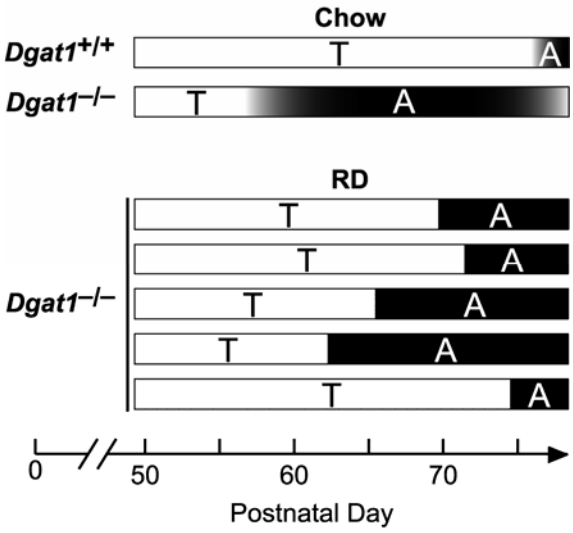

Figure 6. Retinoid deprivation modulates the onset of the second anagen phase in *Dgat1*$^{-/-}$ mice. Schematic timeline depicting the onset of the second postnatal anagen phase in 5 individual retinoid depleted *Dgat1*$^{-/-}$ mice compared to the average age of onset of second anagen in chow fed *Dgat1*$^{-/-}$ and wild-type mice[36]. T, telogen (white bar) and A, anagen (black bar).

DGAT1 protects against retinol toxicity

Because the formation of retinyl esters may help to prevent toxicity due to excessive levels of unesterfied retinol, we examined whether DGAT1 deficiency in skin epidermis resulted in increased susceptibility to retinol toxicity. To test this, retinol or vehicle was applied daily for three days to dorsal cephalad and caudal skin, respectively, of shaved *Dgat1*$^{-/-}$ and wild-type mice. *Dgat1*$^{-/-}$ mice treated with retinol exhibited severe skin irritation characteristic of retinoid toxicity, including erythema, crusty lesions, and severe skin scaling and cracking[33], whereas wild-type skin exhibited only signs of mild irritation (erythema and some skin flaking) (Fig. 7). Skin irritation was not seen in vehicle-treated skin.

The increased susceptibility of *Dgat1*$^{-/-}$ skin to retinol toxicity suggested that the retinol esterification activity of DGAT1 may be cytoprotective. We therefore examined retinol-induced cytotoxicity in *Dgat1*$^{-/-}$ mouse embryonic fibroblasts (MEFs), which have

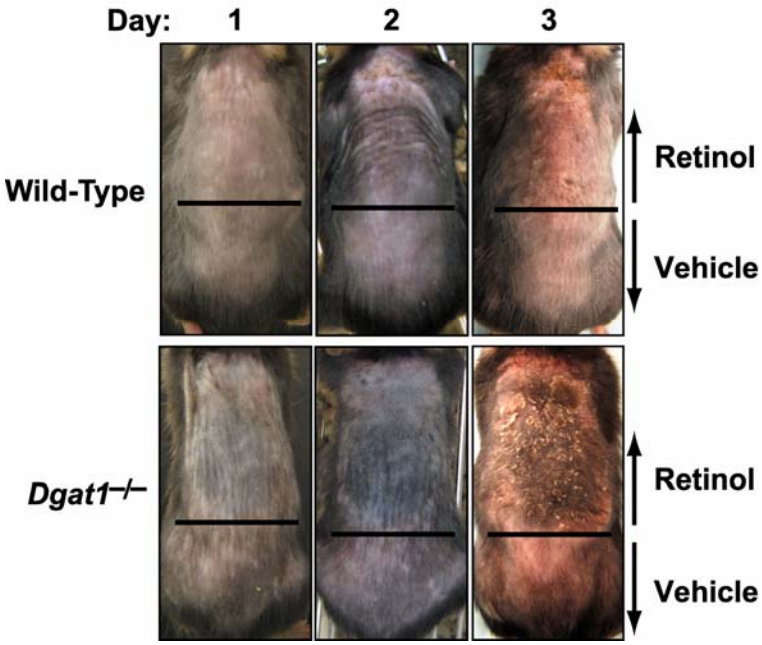

Figure 7. Increased susceptibility to retinol-induced toxicity in *Dgat1*$^{-/-}$ skin. (a) Increased susceptibility of *Dgat1*$^{-/-}$ skin to retinol-induced irritation. Retinol (100 µl of 1 nmol/µl in ethanol) was applied topically once daily for 3 consecutive days to the dorsal cephalad skin. Ethanol alone was applied to the dorsal caudal skin (age 11 weeks, $n = 5$ per genotype).

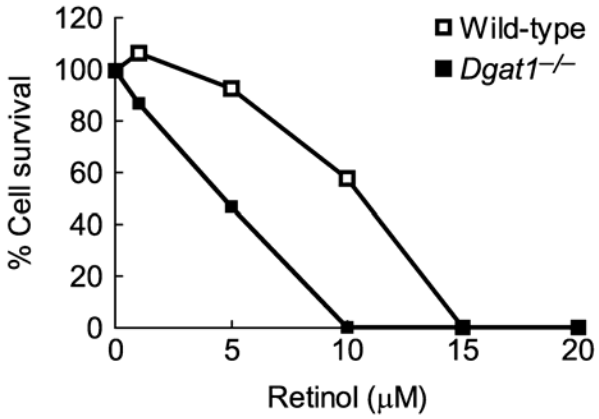

Figure 8. Increased susceptibility of *Dgat1*$^{-/-}$ MEFs to retinol-induced cell death. Cell survival after 24 hours of retinol treatment at indicated concentrations was measured as described in Methods. The experiment was repeated and similar results were obtained.

markedly reduced ARAT activity[25]. Cultured *Dgat1*$^{-/-}$ MEFs exhibited more cell death than wild-type MEFs over a range of retinol concentrations, and the concentration of retinol

required to kill 50% of cells was reduced by ~50% (Fig. 8). In contrast, *Dgat1*$^{-/-}$ MEFs were relatively protected from palmitate-induced cell death (in preparation, R. Streeper and R. Farese, Jr.).

DGAT1 deficiency in the epidermis is sufficient to cause alopecia in mice

To determine whether the retinoid toxicity–associated alopecia in *Dgat1*$^{-/-}$ mice was due to loss of DGAT1 function in the epidermis, we examined mice lacking *Dgat1*

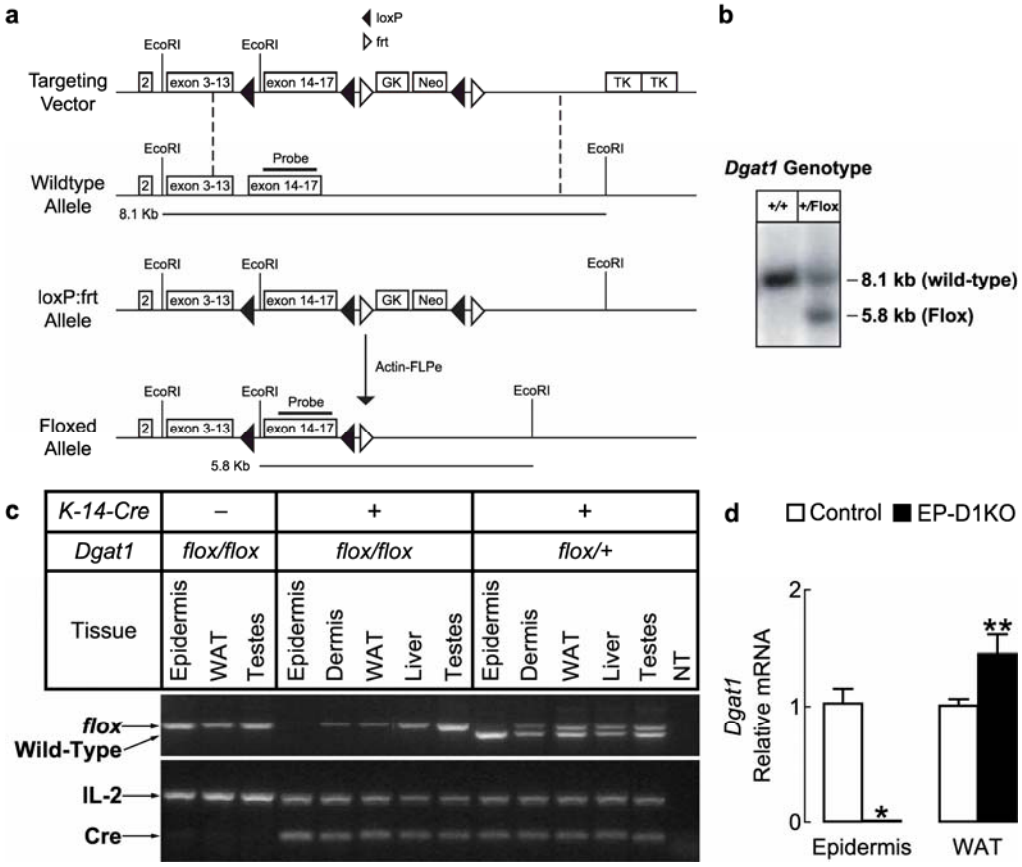

Figure 9. Generation of epidermis-specific *Dgat1*$^{-/-}$ mice. (a) Gene strategy used to generate *Dgat1*$^{flox/flox}$ mice. *Dgat1*$^{flox/flox}$ mice have exon 14-17 flanked by loxP sites. (b) Southern blot demonstrating disruption of *Dgat1* locus. (c) Specific deletion of *Dgat1* in epidermis. PCR detection of the wild-type and floxed alleles of *Dgat1* and Cre transgenes in genomic DNA. PCR detection of IL-2 served as an internal PCR control. Absence of PCR band indicates Cre-mediated recombination. (d) Absence of *Dgat1* mRNA in the epidermis of *K14-Cre*$^{+}$*Dgat1*$^{flox/flox}$ (EP-D1KO) mice. mRNA levels of *Dgat1* in the tail epidermis and white adipose tissue (WAT) were quantified by real-time PCR. (age 13 weeks, n = 3–6 mice in each group). ***P* < 0.001 and ****P* < 0.05 *versus* control.

specifically in the epidermis. To generate epidermis-specific *Dgat1*$^{-/-}$ mice (EP-D1KO mice) we crossed *Dgat1*$^{flox/flox}$ mice with transgenic mice expressing Cre recombinase under the human keratin 14 promoter(Fig. 9a-b).[37] PCR of DNA from wild-type and EP-D1KO mice demonstrated specific deletion of the floxed *Dgat1* allele in the epidermis (Fig.9c), and *Dgat1* mRNA levels were absent from the epidermis of EP-D1KO mice (Fig. 9d). *Dgat1* mRNA expression was modestly increased in the reproductive fat pad of EP-D1KO mice for unknown reasons (Fig. 9d).

EP-D1KO and control mice were observed for signs of alopecia from birth until 15 weeks of age. Whereas control mice did not develop alopecia (Fig. 10a), EP-D1KO mice developed prominent alopecia as early as 7 weeks of age (Fig. 10a). Associated with this, the mRNA levels of RA target genes *CrbpI* and *CrabpII* were ~4.5-fold and ~3-fold higher, respectively, than levels in the skin of control mice (Fig. 10b). These data show that deficiency of DGAT1 specifically in the epidermis was sufficient to cause alopecia and alter retinoid activity in the skin of mice.

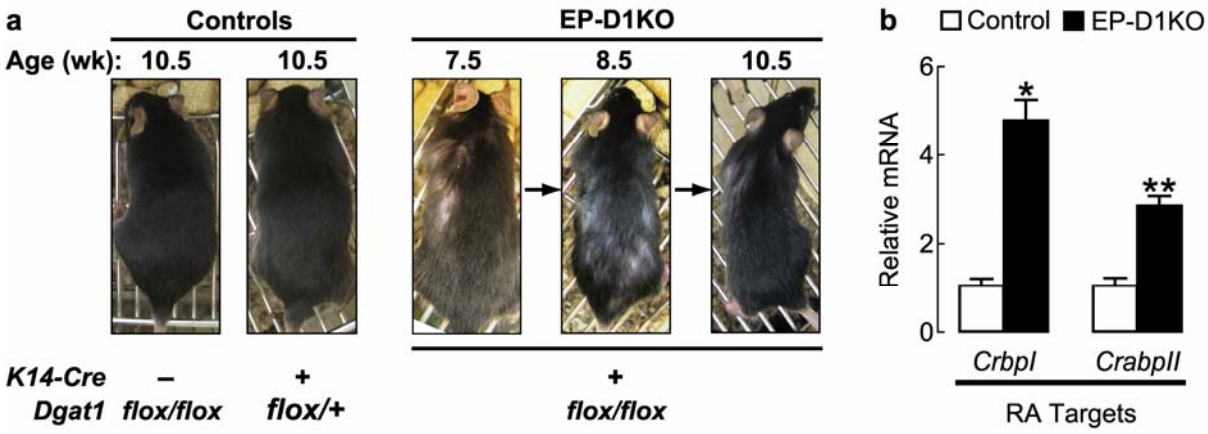

Figure 10. The effects of *Dgat1* deficiency on retinoid homeostasis are epidermis autonomous. (a) Alopecia in EP-D1KO mice. Alopecia was detectable by 7.5 weeks of age. Control mice did not develop detectable alopecia. (n = 6 per genotype observed) (b) Increased mRNA expression of RA target genes in whole skin of EP-D1KO mice. mRNA levels were quantified by real-time PCR. (age 13 weeks, n = 5 per genotype). *P < 0.0001 and **P = 0.0002 *versus* control.

Discussion

In this study, we show that *Dgat1* encodes an ARAT that is crucial for maintaining retinoid homeostasis in the epidermis. This function of *Dgat1* is of significance in regulating hair growth, as *Dgat1* inactivation alters hair cycling in a retinol-dependent manner. This is the first demonstration that the multifunctional enzyme encoded by *Dgat1*[25,26], previously shown to be important in triglyceride and energy metabolism[27], plays a physiological role in retinol metabolism. Further, we identify *Dgat1* as a key modulator of hair cycling.

Studies of mice lacking LRAT revealed its function as the predominant retinol acyltransferase for maintaining adequate stores of retinol in the body[17-19], particularly in the liver where most retinol is stored. Our findings that *Dgat1* inactivation did not predispose mice to retinol deficiency support this conclusion. Levels of retinyl esters in the liver and other tissues (WAT, brown adipose tissue, skeletal muscle, and brain; not shown) and serum retinol levels were normal in *Dgat1*$^{-/-}$ mice fed either a RS or RD diet.

In contrast to the function of LRAT, our study suggests that the ARAT encoded by *Dgat1* functions in the epidermis primarily to prevent retinoid toxicity, likely by helping to limit RA concentrations by controlling the concentration of retinol. The *Dgat1*-encoded enzyme accounted for most of the ARAT activity in skin, and *Dgat1* inactivation did not lower levels of skin retinyl esters but did predispose to elevated levels of retinol and RA when retinol was abundant in the diet. The cyclical alopecia and excessive hair shedding were likely due to excessive retinoid action, since reducing retinol levels in the skin by depriving mice of retinol in their diet largely prevented these phenotypic manifestations. Further, repletion of retinol in the diet of retinol-deprived mice induced hair loss. The alopecia resulting from *Dgat1* inactivation was tissue autonomous, since the phenotype was

present in mice lacking the enzyme specifically in the epidermis. These findings indicate a physiologic role for *Dgat1* in maintaining retinoid homeostasis by preventing retinoid toxicity in the epidermis. Supporting the idea that ARAT activity may prevent retinoid toxicity, previous studies have shown that the K_m for retinol as a substrate in the ARAT reaction is higher than that for the LRAT reaction[38], and ARAT utilizes predominantly retinol that is unbound to intracellular binding proteins as a substrate[22].

Our findings that *Dgat1*$^{-/-}$ skin exhibited more severe skin irritation in response to topically applied retinol than wild-type skin also indicate that *Dgat1* protects against the development of retinoid toxicity, most likely by preventing the accumulation of unesterified retinol, which would drive the biosynthesis of RA and perhaps other active retinoids.[39,40] A cytoprotective role for *Dgat1* was also found in our studies of *Dgat1*$^{-/-}$ MEFs, which exhibited more cytotoxicity than wild-type MEFs in response to retinol treatment. Supporting a role for retinol esterification as a means of preventing retinoid toxicity, studies in human skin and cultured keratinocytes show that increasing retinol concentrations elicits an increase in retinol esterification without increasing RA levels[41,42]. Furthermore, the administration of exogenous RA to cultured human keratinocytes increases conversion of retinol into retinyl esters and reduces endogenous RA production.[39] We observed not only an increase in RA in skin of *Dgat1*$^{-/-}$ mice, but also an increase in expression of RA-regulated genes. This shows that the increase in RA has biological consequences. Notably, *CrabpII* expression increased in the *Dgat1*$^{-/-}$ mice. This is significant because *CrabpII* delivers RA to RAR in the nucleus and thereby sensitizes cells to RA.[43] Thus, the increase in both RA and *CrabpII* in the *Dgat1*$^{-/-}$ mice likely contributes to the retinoid-toxicity phenotype.

Retinoids exert diverse effects on hair, including promoting hair loss and, paradoxically, hair growth.[44,45] We show that *Dgat1*, by modulating retinoid homeostasis, is a key regulator of hair cycling in mice. Mice that lack *Dgat1* exhibited altered hair cycling, characterized by an increased propensity of follicles to be in anagen (Chapter 2). These findings are consistent with a previous study in mice showing that topical retinoids applied to telogen hair follicles rapidly stimulated and prolonged anagen.[45]

Precisely how retinoids regulate hair cycling is currently unclear. However, several important molecules of retinoid metabolism [*e.g.*, CRABPII, retinol dehydrogenase (Dhrs9), and aldehyde dehydrogenases (Aldh1a1–3)] and retinoid signaling (RARβ and RARα) exhibit spatiotemporal expression patterns in the hair follicle that are cycle-dependent, suggesting that local retinoid production and signaling is required in different structures of the follicle at each stage of the cycle.[14,46] Interestingly, we found in Chapter 2 that *Dgat1* exhibited a cycle-dependent pattern of expression, with the highest mRNA levels during telogen. Taken together, our findings suggests a role for *Dgat1* in maintaining the follicle in the telogen phase; in the absence of *Dgat1*, there is an increased propensity of hair follicles to enter anagen .

The mechanisms of retinoid-induced alopecia are not well understood. Studies of human hair suggest that the retinoids induce telogen effluvium and predispose to increased shedding of telogen hairs.[47,48] However, whether the shedding of telogen hairs occurs passively or involves an active mechanism of controlled signal-induced release of the hair shaft is unknown. In our study (Chapter 2), increased shedding of telogen hairs was associated with the onset of anagen, suggesting the intriguing possibility that enhanced retinoid action might affect exogen, a postulated active mechanism of hair shedding that is

linked to anagen.[49] In this respect, it will be of interest in future studies to determine whether inactivation of *Dgat1* promotes premature entry into exogen.

Although the RD diet normalized all-*trans*-RA levels in the skin and prevented alopecia in *Dgat1*$^{-/-}$ mice, it did not completely normalize the increased RA target gene expression or prevent the precocious onset of second anagen. A possible explanation for this is that all-*trans*-RA levels may have remained increased in specific sub-compartments of the skin, but these increases were not detectable in whole skin. Alternatively, other biologically active retinoids, such as 3,4-*didehydro*-retinoic acid, which are present in skin[13] but were not measured in our studies, may have been increased in *Dgat1*$^{-/-}$ skin.

Finally, our study shows that at least one aspect of the pleiotropic phenotype of murine *Dgat1* inactivation, *i.e.*, alopecia, results from changes in retinoid metabolism. It will be of interest to determine if other aspects of the *Dgat1* knockout phenotype are similarly related to retinoid metabolism. For example, *Dgat1*$^{-/-}$ mice exhibit increased insulin sensitivity.[28] Because levels of circulating retinol binding protein 4 have been linked to insulin resistance[50], this raises the possibility that changes in glucose metabolism in *Dgat1*$^{-/-}$ mice may be retinol dependent. Although we do not yet know whether the alterations in hair biology that result from DGAT1 deficiency are relevant for humans, our results are pertinent to existing pharmaceutical strategies to develop DGAT1 inhibitors for the treatment of metabolic diseases. In addition, our findings may have implications for the treatment of disorders of hair growth, such as alopecia or hirsutism.

Methods

Mice and diets. Male *Dgat1*$^{-/-}$ mice and wild-type mice were of C57BL/6J genetic background and genotyped as previously described.[27] Mice were housed in a pathogen-free barrier facility (12-h light/12-h dark cycle) and fed retinoid-abundant chow (5053 PicoLab Diet; Purina, St. Louis, MO) unless otherwise specified. For studies in which dietary retinol content was controlled, *Dgat1*$^{+/-}$ dams were fed a RD diet containing 10 kcal% fat and <0.04 IU/g of retinol (D03102201; Research Diets, New Brunswick, NJ) throughout gestation and suckling. Offspring of dams fed a RD diet are born with reduced hepatic retinoid stores, and continuing this diet after weaning allows for gradual depletion of both hepatic and extra-hepatic tissue stores. The first litter of *Dgat1*$^{-/-}$ and wild-type offspring were weaned and maintained on the RD diet until 3 weeks prior to sacrifice when the diet was changed to a RS diet containing 10 kcal% fat and 4 IU/g of retinyl palmitate (D12450B; Research Diets, New Brunswick, NJ). To generate RD mice, the second litter of RD *Dgat1*$^{+/-}$ dams were weaned and maintained on the RD diet.

To generate *Dgat1*$^{flox/flox}$ mice a targeting vector was designed to flank exons 14–17 with loxP sites, a region similar to that deleted in *Dgat1*$^{-/-}$ mice [1]. *Dgat1* genomic fragments were amplified by PCR from 129/SvJae mouse genomic DNA. The vector was constructed in the pJB1 vector (gift from Dr. Joachim Herz, University of Texas Southwestern Medical Center, Dallas, TX) by subcloning a 1.0-kilobase (kb) upstream short-arm fragment containing 5′-coding sequences (sense primer 5′-cggggtaccGCTTTATTCCTACCGGGATG-3′ and antisense primer 5′-aagcggccgcAAACAATGGGATAAGCACAG-3′), a 817-bp fragment containing exons 14–17 of Dgat1 (sense primer 5′ggaattcCTGTGCTTATCCCATTGTTT-3′ and antisense primer

5′-gccggtaccAAATGCCATCCCCAAGAGCA-3′), and a 7.5–kb downstream long-arm fragment containing the *Dgat1* stop codon and polyadenylation signal (sense primer 5′-ccctcgagGGCATTTGAATCTCACCACTG-3′ and antisense primer 5′-ccgctcgagTCAGCTGATTGGTCTTCACAC-3′). Primer sequences (lowercase letters) were added on the primer termini to introduce *Kpn*I (short arm sense primer), *Not*I (short arm antisense primer), *EcoR*I (exons 14–17 sense primer), *Kpn*I (exons 14–17 antisense primer), and *Xho*I (long arm sense and antisense primers) restriction enzyme sites for cloning. The targeting construct was introduced into 129/SvJae murine embryonic stem cells (line RF8), and clones containing targeted alleles were identified by PCR and verified by Southern blotting. Cells harboring the targeted *Dgat1* allele were used to generate mice, and subsequent genotyping in mice was performed by PCR. Heterozygous $Dgat1^{flox:frt/+}$ mice were then crossed with Actin-FLPe transgenic mice obtained from Jackson Laboratory in a C57BL/6J genetic background to remove the neomycin resistance gene. The FLPe transgene was genotyped by PCR using sense primer 5′-CACTGATATTGTAAGTAGTTTGC-3′ and antisense primer 5′-CTAGTGCGAAGTAGTGATCAGG-3′. For Southern blotting, the floxed *Dgat1* allele was confirmed by hybridizing *EcoR*I-digested genomic DNA with a 32P-labelled 822-bp fragment containing exons 14–17 of *Dgat1* amplified by PCR from the genomic DNA using the sense primer 5′-CTGTGCTTATCCCATTGTTT-3′ and antisense primer 5′-AAATGCCATCCCCAAGAGCA-3′. The wild-type *Dgat1* allele yields an 8.1–kb fragment and the floxed *Dgat1* allele a 5.8–kb fragment. A further cross was carried out with $Dgat1^{+/+}$ mice. Pups carrying the floxed allele of *Dgat1* and not Actin-FLPe were selected to generate $Dgat1^{flox/flox}$ mice (mixed C57BL/6 and 129/SvJae background) to cross with Keratin 14-Cre mice (K14-Cre; mixed Swiss Webster;129/Sv; C57BL6/J; CBA/J;

Jackson Laboratory, Bar Harbor, Maine).[37] *K14-Cre*$^{+}$*DGAT1*$^{flox/+}$ mice were crossed with *Dgat1*$^{flox/flox}$ mice to generate male *K14-Cre*$^{+}$*DGAT1*$^{flox/+}$, *K14-Cre*$^{+}$*DGAT1*$^{flox/flox}$, and *Dgat1*$^{flox/flox}$ littermates (mixed Swiss Webster;129/Sv; C57BL6/J; CBA/J) for studies. All experiments were approved by the Committee on Animal Research of the University of California, San Francisco.

Genotyping of *Cre* and floxed *Dgat1* alleles. Genomic DNA was extracted from tail epidermis (the tail epidermis was separated from the dermis mechanically after incubation of skin at 37°C for 45 minutes) and dermis, WAT, liver, and testes. The presence of *Cre* was detected with sense primer 5′-GCGGTCTGGCAGTAAAAACTATC-3′ and antisense primer 5′-GTGAAACAGCATTGCTGTCACTT-3′ (100-bp product) and interleukin-2 (IL2) by sense primer 5′-CTAGGCCACAGAATTGAAAGATCT-3′ and antisense primer 5′-GTAGGTGGAAATTCTAGCATCATCC-3′ (324-bp product) served as an internal PCRcontrol. To identify various alleles of *Dgat1,* genomic PCR was performed with forward primer 5′-CAGACATGGCAGCAGCAAATG-3′ (located in exon 15) and reverse primer 5′-TGCAAGTTGCTGCTGCCACCTG-3′ (located in the 3′ un-transcribed region). The wild-type *Dgat1* allele yields an 895-bp band, and the floxed *Dgat1* allele a 1002-bp band. Absence of the 1002-bp band indicates Cre-mediated recombination of floxed *Dgat1* allele and the deletion of exons 14–17.

Retinol esterification assays. Mouse skin was homogenized with a Tissue Tearor^{TM} (Model 398, Probe 9853G-04, Biospec Products, Inc., Bartlesville, OK) in 50 mM Tris-

HCl (pH 7.4) and 250 mM sucrose containing proteinase inhibitors (Roche Diagnostic, Mannheim, Germany). ARAT assays were performed with 100 μg of total protein homogenates in an assay mix containing Buffer A [5 mM $MgCl_2$, 1.25 mg/ml BSA, 200 μM all-*trans*-retinol (Sigma-Aldrich, St. Louis, MO) in acetone, and 25 μM [^{14}C]oleoyl CoA (55.0 mCi/mmol)]. After 10 min at 37ºC, lipids were extracted with chloroform:methanol (2:1, v:v) and separated by silica gel G-60 TLC plates with hexane:ethyl ether:acetic acid (80:20:1). Retinyl ester, triacylglycerol, and cholesterol ester bands were scraped, and radioactivity was measured by scintillation counting.

Retinoid analyses. ROL, RE and all-*trans*-RA were quantified as described[51], with modifications. Mouse tissue samples were harvested under yellow light and immediately frozen in liquid N_2. Tissues were homogenized on ice with ice-cold 0.9% saline to make a ~25% homogenate. Tissues were homogenized using ground glass vessels (Kontes, size 21) either manually or with a Heidolph motorized homogenizer (at 280 RPM). For skin samples (200-400 mg), a portion (50-100 mg) would not homogenize and was subtracted to obtain a tissue mass. Serum was obtained by centrifuging blood at 7000 g for ~7 min at 4^{o}C. Tissue homogenate or serum was added to a disposable glass culture tube (16x150 mm), an internal standard (50-100 nM 4,4-dimnethyl-RA delivered in 10 μL of acetonitrile) was added, followed by addition of 0.025 M KOH in ethanol (1 ml for serum, 3 ml for tissue homogenates), and extraction with 10 ml hexane. The organic phase containing nonpolar retinoids (ROL and RE) was removed. 4 M HCl (60-180 μL) was added to the aqueous phase, and polar substances (RA) were removed by extraction with 10 ml hexane. Organic phases were dried under nitrogen with gentle heating at ~25-30^{o}C in a water bath

(Organomation Associates Inc. model N-EVAP 112, Berlin, MA). Extracts were resuspended in acetonitrile according to analyte: (RA in 60uL, retinol/retinyl ester from liver in 1000 μL, retinol/retinyl ester from all other tissues in 150 μL.) Only glass containers, pipettes, and calibrated syringes were used to handle retinoid samples.

Real-time PCR. Whole skin was homogenized and total RNA extracted with RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA) RNA (5 μg) was reverse-transcribed using the Superscript III™ First-Strand Synthesis Supermix kit (Invitrogen, Carlsbad, CA). Real-time PCR was performed and analyzed with the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Each 20-μl PCR reaction contained 2 μl of cDNA, 10 μl of 2x SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) and10 pmol each of forward and reverse primer. Relative expression levels were calculated by the comparative C_T (cycle of threshold detection) method as outlined in the manufacturer's technical bulletin. Cyclophilin expression was used as control. The primers used for RT-PCR are listed in Table 1.

Table 1: **Primer Sequences for Real-time PCR**

Gene	Sense	Antisense
Dgat1	5′-TTCCGCCTCTGGGCATT-3′	5′-AGAATCGGCCCACAATCCA-3′
CrabpII	5′-CCTCCTGGAGCCGAGAACT-3′	5′-GGTGCACACAACGTCATCTG-3′
Crbpl	5′-GATGAACTTCACCTGGAAATGAGA-3′	5′-GGGCTGCTCAGTGTACTTTCTTAA -3′
β-*catenin*	5′-TTAAACTCCTGCACCCACCAT-3′	5′-CTAGTCGTGGAATAGCACCCTGTT-3′
Vegf	5′-GCAGGCTGCTGTAACGATGAA-3′	5′-TGAGGTTTGATCCGCATGATC-3′
Cyclophilin	5′-TGGAAGAGCACCAAGACAGACA-3′	5′-TGCCGGAGTCGACAATGAT-3′

Cell survival assay with mouse embryonic fibroblasts

MEFs were cultured from *Dgat1*$^{-/-}$ embryos and their wild-type littermates as described in [52] and cultured in knockout Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 50 units/ml penicillin and 50 units/ml streptomycin. Primary MEFs (passage 2 or 3) were seeded in triplicate into 96-well plates, and cells at 90% confluency were treated with retinol dissolved in DMSO. FBS concentration was reduced to 1% at the start of treatment. Cell survival was measured 24 hours later using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI).

Topical retinol treatments. All-*trans*-retinol (Sigma-Aldrich, St. Louis, MO) was dissolved in ethanol (1 nmol/μl) and 100 nmol was applied topically to the skin of shaved mice ($n = 5$ per group) daily for three consecutive days. As a control, ethanol was applied to skin of the same mice.

Statistical analyses. Values are reported as mean ± *s.e.m*. Means were compared with either a Students *t*-test or ANOVA followed by the Tukey or Bonferroni test.

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CHAPTER IV.

Conclusions

Implications

Despite significant progress towards understanding whole animal retinoid homeostasis, substantially less is known about the factors that regulate retinoid homeostasis at the target tissue level. The skin is perhaps one of the most well-studied retinoid target tissues.[1,2] Significant advances have been made towards identifying the effects of exogenous retinoids on skin biology and the mechanisms of retinoid action, but many questions still remain about the enzymes and the mechanisms that regulate retinoid homeostasis in the skin. In particular, the mechanisms that regulate the uptake and metabolic partitioning of retinol - either for storage or for oxidation to form RA for signaling or excretion - are largely unknown. The regulation of enzymes that synthesize RA from retinol, *i.e.* retinol and retinal dehydrogenases, is not well understood due to difficulties overcoming the immense functional redundancy that exists for these enzymatic activities *in vivo*.[3] The work presented in this thesis offers some new insights toward an additional mechanism that regulates retinoid homeostasis in the skin.

We show that retinol esterification plays an important role in maintaining retinoid homeostasis in the skin. Specifically, we show that the enzyme encoded by *Dgat1* functions as a retinol acyltransferase in the skin, where it helps to maintain retinoid homeostasis by preventing retinoid toxicity. In mice lacking *Dgat1*, feeding a retinol-abundant diet resulted in increased retinol and all-*trans*-RA levels, and elevated retinoic acid activity in the skin. The excess retinoids and retinoic acid activity in the skin have significant biological consequences including altered hair cycling and cyclical alopecia due to excessive hair shedding because feeding a retinoid-deficient diet reduced, in the skin, retinol and all-*trans*-RA levels, retinoic acid activity, and protected against alopecia. Mice lacking *Dgat1* were

also more susceptible to the toxic effects of topical retinol application. Furthermore, we found that the retinol acyltransferase function of *Dgat1* is epidermis-autonomous, since the specific deletion of *Dgat1* in the epidermis was sufficient to cause alopecia. This is the first demonstration that the multifunctional enzyme encoded by *Dgat1*, previously shown to be important in triglyceride and energy metabolism[4-6], plays a pivotal role in retinoid metabolism. This is also the first demonstration that retinol esterification in the skin functions to maintain retinoid homeotasis by restriction RA biosynthesis through controlling retinol levels. Whether this function of retinol esterification is important in other retinoid tissues besides skin is currently unknown.

The physiologic significance of enzymes that recognize "free" retinol, *i.e.* retinol not bound to CRBP, as substrate has been controversial. This debate has been ongoing since the discovery that several isoforms of CRBPs are highly expressed in almost all tissues of humans, rats, and mice.[7] Since the expression of CRBPs greatly exceeds the intracellular concentration of retinol, it was postulated that the concentration of "free" retinol in cells is unlikely to be significant[8] and CRBP-bound retinol is the more physiological substrate for retinol metabolizing enzymes *in vivo*.[7] This argument shifted scientific interest and resources away from many retinol metabolizing activities that were historically studied, including the ARAT activity, to focus mainly on retinol metabolizing activities that recognize CRBP-bound retinol as substrate. Our finding that the DGAT1 enzyme, an enzyme which only recognizes "free" retinol as substrate, plays an essential role in the maintenance of retinoid homeostasis in the skin, implies that "free" retinol is a physiologically relevant substrate and that the metabolism of "free" retinol and the enzymatic activities that utilize "free" retinol as substrates likely play important roles in regulating the biological activity of retinol *in vivo*.

Future perspectives

Does leptin modulate retinol metabolism in the skin? Leptin-deficiency eliminates the metabolic benefits as well as the alopecia and sebaceous gland defects of *Dgat1*$^{-/-}$ mice.[9,10] It is logical then to consider the possibility that leptin-deficiency prevents the development of retinoid toxicity from *Dgat1* deficiency in the skin. Leptin may repress the expression of other ARAT enzymes in the skin and in the absence of leptin, upregulation of other ARAT activities may compensate for the loss of the *Dgat1* encoded ARAT enzyme. At least one other ARAT enzyme is known to exist in the skin. We recently identified a multifunctional O-acyltransferase (MFAT) enzyme that is highly expressed in the skin.[11] In insect cells and mammalian cells overexpressing MFAT, an increase in acyl-CoA:retinol acyltransferase activity was detected.[11] Whether leptin regulates the expression of the MFAT enzyme or other ARAT enzymes in the skin is unknown. Future studies will also be necessary to determine whether the reversal of the metabolic phenotype of *Dgat1*$^{-/-}$ mice by leptin deficiency is partially retinoid related. These studies may reveal an important cross-talk between the leptin and retinoic acid signaling pathways in the regulation of energy homeostasis.

Is the impaired mammary gland phenotype of Dgat1$^{-/-}$ *mice related to the ARAT activity of DGAT1?* ARAT activity has been detected in both the stromal and epithelial compartments of the mammary gland.[12] Retinoic acid signaling has been shown to be required for regulating both proliferation and differentiation of the mammary epithelium during mammary gland morphogenesis.[13] The defect in the mammary gland development of *Dgat1*$^{-/-}$ mice is characterized by decreased epithelial proliferation and reduced markers of functional differentiation resulting from *Dgat1* deficiency in both the stromal and epithelial

compartments of the gland.[14] Whether ARAT activity is reduced in the mammary glands of *Dgat1*$^{-/-}$ mice and whether altered retinoid metabolism plays a role in the mammary gland defects is currently unknown.

Are aspects of the metabolic phenotype of Dgat1$^{-/-}$ *mice retinoid related?* Recently, elevated levels of circulating retinol binding protein 4 (RBP4) have been linked to insulin resistance [15]. The RBP4 protein is synthesized and secreted by hepatocytes and adipocytes and functions to deliver retinol to tissues[16]. Secretion of RBP4 bound retinol by hepatocytes and adipose tissue are normally under tight homeostatic control.[17] The overproduction and secretion of RBP4 in insulin-resistant mice and humans suggest that altered retinoid homeostasis in the WAT may contribute to the development of insulin resistance. *Dgat1*$^{-/-}$ mice exhibit increased insulin sensitivity on a high-fat diet[18]. Preliminary measurements of retinoid levels in the WAT of chow fed *Dgat1*$^{-/-}$ mice did not reveal significant alterations in retinoid levels (Table 1; appendix), however, it will be of interest to measure retinoid levels in the WAT and insulin sensitive tissues of the insulin-sensitive high-fat diet fed *Dgat1*$^{-/-}$ mice. Retinoid deprivation of high-fat diet fed *Dgat1*$^{-/-}$ mice may also be useful for assessing the role of retinoids in the enhanced insulin sensitivity of *Dgat1*$^{-/-}$ mice and provide new insights into the role of retinoids in the mechanisms of insulin sensitivity.

Does the enzyme encoded by Dgat1 *function as a retinol acyltransferase enzyme in the brain?* The role of retinoids in the adult nervous system has only recently gained importance and scientific attention. Studies in retinoid-depleted rats and mice and retinoid receptor knockout mouse models have revealed functional roles for RA in a number of neural processes and implicated altered RA signaling in the development of a number of neurological diseases. For example, retinoids are implicated in the mechanisms of learning

and memory involving effects on neural plasticity and LTP in the hippocampus.[19,20] Retinoid depleted rats and mice, rats that were fed a retinoid-deficient diet for 12 weeks and mice that were fed a retinoid-deficient diet for 39 weeks, exhibited deficits in spatial learning which were reversed by administration of all-*trans*-RA.[21] A decline in retinoid receptor expression in the brain was also associated with age-related impairments in the memory performance of mice. These impairments were reversed by RA injections that enhanced retinoid receptor expression in the brain and improved memory performance.[22]

In the corpus striatum, RA may also function in the control of motor coordination and locomotor activity by directly regulating the expression of the dopamine D2 receptor via an RARE in the promotor.[23,24] Retinoid receptor knockout mice exhibit locomotor deficits that are accompanied by a decrease in the expression of the dopamine D2 receptor in the striatum. Reduced retinoid signaling in the brain, which was inferred from the decrease in expression of a large number of RA target genes in the brain, is also implicated in several motor disorders including Parkinson's disease, motoneuron disease, and Huntington's disease.[25-32] RA signaling is involved in a number of other neural processes and neurological diseases but are beyond the scope of this thesis.

The specific functions of RA the CNS are currently unclear but many components of the Ca^{2+}-dependent second-messenger systems, neurotransmitter- and neurotrophin-signaling pathways are gene targets of RA.[33] Non-classical modes of RA action involving interactions with enzymes including protein kinase C and direct activation of electrical synapses may also be involved.[33]

Studies in rats suggest that the CNS synthesizes RA more efficiently than other target tissues. RA comprises a greater proportion of the retinoid pool in the CNS compared with

other retinoid target tissues and this was not a result of increased RA transport from the blood to the brain. Components of the retinol metabolism system including retinoid binding proteins, RA synthesis enzymes, and RA metabolizing enzymes are highly expressed in the adult brain, suggesting similar mechanistic controls of retinoid homeostasis and action in peripheral tissues are also at work in the brain.

Interestingly, *Dgat1* mRNA is highly expressed in many regions of the brain we examined including the hypothalamus, cerebellum, cortex, and hippocampus, (MYS Shih and RV Farese Jr., unpublished data) although very little triacylglycerol is found in the brain. It is likely that DGAT1 functions as an ARAT enzyme in the brain. Although our initial measurements of retinoid levels in the whole brain of *Dgat1*$^{-/-}$ mice and did not reveal remarkable alterations in retinoid levels compared with wild-type mice (Table 1; appendix), the possibility exists that retinoid metabolism and retinoid levels may be altered in localized brain structures that were not assessed in our study. Further examination of retinoid levels in specific brain structures may reveal novel retinoid related roles for DGAT1 in the CNS.

Of note, our study confirms, for the first time, that retinoic acid levels in the brain can be dramatically depleted by dietary retinoid deprivation. We measured retinoid levels in the whole brain of mice born from dams that were fed retinoid-deficient diet during the gestational period. We found that retinyl ester and retinol concentrations in the whole brain of the wild-type retinoid-deficient (RD) mice were reduced by ~70% and ~90%, respectively compared with levels in the brains of the retinoid-sufficient (RS) mice. (Table 1;appendix). Most importantly, we demonstrated that dietary retinoid deprivation reduced all-*trans*-RA levels to ~53% of normal levels found in retinoid-sufficient brains (RS: 67.208 ± 0.368 vs RD: 34.960 ± 11.616 *pmol/g*, $P<0.05$).

Does altered retinoid homeostasis in the CNS of Dgat1$^{-/-}$ mice play a role in the enhanced leptin sensitivity of Dgat1$^{-/-}$ mice? Recently, synaptic plasticity was identified as a major mechanism by which peripheral metabolic hormones, including leptin, ghrelin, and estrogen, influence energy balance.[34] Rapid synaptic remodeling within the hypothalamic arcuate nucleus was detected after delivery of leptin to *ob/ob* mice, normalizing both excitatory and inhibitory post-synaptic densities toward normal values 6 hours prior to observed effects on food intake.[35] Interestingly, *Dgat1*$^{-/-}$ mice exhibit enhanced leptin sensitivity which may be related to *Dgat1* deficiency in the CNS. It is currently unknown if the enhanced leptin sensitivity of *Dgat1*$^{-/-}$ mice results from increased activation of downstream signal transduction pathways or enhanced effects of leptin on the rewiring of hypothalamic metabolic circuits. Given RA's role in synaptic plasticity and the presence of retinoid receptors in the hypothalamus, altered retinoid homeostasis in the hypothalamus of *Dgat1*$^{-/-}$ mice may modulate the hypothalamic response to leptin through modulation of synaptic plasticity.

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Appendix

Table 1. Retinoid levels in tissues of wild-type and *Dgat1*$^{-/-}$ mice fed a retinoid-suffient or retinoid-deficient diet

	Retinol				**Retinyl Esters**				**All-*trans*-Retinoid acid**			
	Sufficient		**Deficient**		**Sufficient**		**Deficient**		**Sufficient**		**Deficient**	
***Dgat1* genotype**	**+/+**	**–/–**	**+/+**	**–/–**	**+/+**	**–/–**	**+/+**	**–/–**	**+/+**	**–/–**	**+/+**	**–/–**
	nmol/g	*nmol/g*	*nmol/g*	*nmol/g*	*nmol/g*	*nmol/g*	*nmol/g*	*nmol/g*	*pmol/g*	*pmol/g*	*pmol/g*	*pmol/g*
Liver	9.331 ± 5.043	9.424 ± 1.816	0.154 ± 0.021*	0.237 ± 0.129*	79.146 ± 52.069	61.501 ± 28.987	2.696 ± 0.928*	3.117 ± 0.466*	61.323 ± 13.362	57.705 ± 17.678	41.623 ± 7.472*	32.685 ± 9.688*
WAT	0.439 ± 0.149	0.450 ± 0.119	0.147 ± 0.111*	0.132 ± 0.026*	0.540 ± 0.065	0.508 ± 0.169	0.647 ± 0.155	0.540 ± 0.134	4.648 ± 0.881**	5.580 ± 1.167	7.006 ± 0.707	6.669 ± 1.438
Serum	1.150 ± 0.231	1.196 ± 0.071	0.215 ± 0.169*	0.197 ± 0.086	0.131 ± 0.014	0.109 ± 0.104	0.105 ± 0.062	0.105 ± 0.095	4.777 ± 0.755	3.688 ± 1.818	4.786 ± 0.845	5.222 ± 1.718
BAT	0.639 ± 0.539	0.468 ± 0.190	0.232 ± 0.037*	0.259 ± 0.187*	0.989 ± 0.501	1.003 ± 0.293	1.125 ± 0.176	0.951 ± 0.442	19.089 ± 7.429	24.079 ± 8.906	12.101 ± 4.360	12.189 ± 7.027
Muscle	0.408 ± 0.145	1.035 ± 0.575	0.033 ±0.017*	0.057 ±0.009*	0.623 ± 0.176	0.965 ± 0.423	0.357 ± 0.057*	0.423 ± 0.139#	3.847 ± 0.616	3.773 ± 0.283	3.199 ± 0.937	2.251 ± 0.519*
Whole Brain	2.111 ± 0.958	1.356 ± 1.144	0.045 ± 0.012*	0.050 ± 0.018*	1.633 ± 0.506	1.624 ± 0.815	0.493 ± 0.047*	0.484 ± 0.055*	67.208 ± 0.368	77.860 ± 8.036	34.960 ± 11.616*	29.045 ± 11.791*

Values are mean ± *s.e.m* from 4 to 6 mice in each group, *P<0.05 *versus* VAS diet, ** P<0.05 versus VAD diet, # P<0.07 versus VAS diet by two-way ANOVA followed by Bonferroni for multiple comparisons.

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