TO THE EDITOR

Enormous effort over the last 3 decades has identified a number of signaling pathways that act on hair follicle stem cells (HFSCs) to promote both quiescence as well as activation (Chan et al., 2004; DasGupta and Fuchs, 1999; Kimura-Ueki et al., 2012; Ming Kwan et al., 2004; Paus and Foitzik, 2004; Plikus et al., 2008; Sugawara et al., 2010; Vauclair et al., 2005; Zhang et al., 2006). With respect to intrinsic mechanisms of HFSC regulation, less is known about the cellular metabolism of individual cell types in the epidermis. In general, it has been presumed that somatic cells use mostly the electron transport chain (ETC) to produce energy from pyruvate that was generated by the uptake and processing of glucose, while early embryonic and cancer cells are thought to also rely on production of lactate from pyruvate. We recently demonstrated that HFSCs balance the production of energy through the ETC with the production of lactate as well (Flores et al., 2017). Previous efforts to define metabolic activities in the epidermis focused on measurements of enzyme activities on entire follicles (Hamanaka et al., 2013; Kloepper et al., 2015). In addition, several studies used transgenic models targeting the entire epidermis (including the follicle) for deletion of ETC components (Hamanaka et al., 2013; Kloepper et al., 2015). Those studies suggested that genetic blockade of the ETC leads to degeneration of the follicle. It is less clear whether inhibition of ETC complexes, as opposed to genetic ablation of ETC, would affect cell metabolism or fate decisions.

In the study by Flores et al. (2017), we deleted Ldhα specifically in HFSCs and found that blocking lactate production prevented activation of HFSCs. In addition, deletion of a protein required to transport pyruvate into mitochondria (Mpc1) in HFSCs led to the increase of lactate production and acceleration of HFSC activation (Flores et al., 2017). Therefore, we hypothesized that pharmacological ETC inhibition would promote HFSC activation because of increased production of lactate.

To determine whether manipulation of ETC activity could affect HFSC activation, we used topical application of various inhibitors of ETC components during a resting phase of the hair cycle. At postnatal day 50, the HF is in telogen, a resting phase where the stem cells of the follicle are quiescent until the start of the next hair cycle at day 70–80 (Greco et al., 2009; Muller-Rover et al., 2001; Paus and Foitzik, 2004). Rotenone, phenformin, and antimycin A are all established inhibitors of Complex I and Complex III, respectively (Graeber et al., 1976). Animals were shaved at postnatal day 47 and treated with the indicated compounds or vehicle on the shaved area every 48 hours for the indicated duration. After 3–4 treatments (8–12 days), animals treated with ETC inhibitors began to show signs of hair cycle activation macroscopically, judged by pigmentation of the skin in black mice, whereas vehicle-treated mice did not show significant pigmentation for at least 20 days (Figure 1a and Supplementary Figure S1a online). As defined previously, the epidermis of murine skin becomes pigmented upon induction of the hair cycle, which is indicative of the generation of melanocytes injecting pigment (melanin) into the keratinocytes, which go on to make the hair shaft, as well as those in the interfollicular epidermis. Therefore, the induction of pigmentation observed after 8–12 days in ETC inhibitor–treated mice was most likely indicative of hair cycle activation induced by this treatment.

To demonstrate that the pigmentation induced by ETC inhibition was in fact due to changes in HFSC activation, tissue was harvested and subjected to pathology. Histological analysis showed that follicles in back skin treated with ETC inhibitors underwent a normal telogen-to-anagen transition (Figure 1b and Supplementary Figure S1b). These findings were in contrast to previous studies showing that transgenic abrogation of the ETC led to HF degeneration (Kloepper et al., 2015).

To determine whether the hair cycle induction driven by ETC inhibition was typical, we measured the thickness of each layer of skin across different stages of treatment. As shown in Figure 1c, all of the ETC inhibitors increased the thickness of the epidermis, dermis, and particularly the hypodermis, suggesting an expansion of the adipocytes. Analysis of ETC-inhibited skin showed an increase in Ki67 in HFSCs a week after treatment, evidence of HFSC activation in response to ETC inhibition (Figure 1d and Supplementary Figure S1d).

To determine whether application of the ETC inhibitors promoted inflammation, which could cloud interpretation of hair cycle data, we assessed various markers of chemokine response and the presence of inflammatory immune cells after treatment. We found no evidence of significant inflammation by these measures in response to ETC inhibition (Supplementary Figure S1d).

To determine the effect on cellular metabolism of ETC inhibition by rotone, phenformin, and antimycin A, we performed two measures of metabolic pathways. First, we quantified lactate dehydrogenase activity on cells isolated from the epidermis treated with

Abbreviations: ETC, electron transport chain; HFSC, hair follicle stem cell

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ETC inhibitors for 48 hours (Figure 2a). Next, we employed metabolomics on sorted HFSCs with and without treatment for either 48 hours or 10 days. These analyses indicated an increase in Ldh activity and lactate levels, as well as several other glycolytic intermediates, in response to ETC inhibition by rotenone, phenformin, and antimycin A (Figure 2b). This is consistent with our previous data showing that deletion of Mpc1 in HFSCs blocked pyruvate entry into mitochondria, leading to increased levels of lactate (Flores et al., 2017).

As mice age, the hair cycle is known to become protracted, such that upon shaving, only portions of the back skin show regrowth of hair within 1–2 months. We treated various batches of aged mice (at least 17 months) for 30 days with ETC inhibitors to determine whether this metabolic manipulation could stimulate the hair cycle even in dormant follicles. We found that topical application of phenformin, rotenone, or antimycin A all led to more complete hair regrowth across the entire back skin on a time course similar to that of younger mice (Supplementary Figure S2a online). As in younger animals, treatment with these ETC inhibitors led to an increase in lactate pool levels, as measured by metabolomics (Supplementary Figure S2b). The results presented here describe a method to promote lactate production and subsequent hair cycle activation. Building on our previous genetic dissection of metabolism and HFSC activation, this work provides a relatively simple route to promote proliferation of HFSCs.

**Animals**

Wild-type male and female 49-day-old C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME) for all topical experiments. All animals were maintained in University of...
Topical inhibitor treatments
All drugs were suspended in DMSO and aliquoted at approximately five times the half maximal inhibitory concentration for the purposes of penetrating the epidermal barrier upon in vivo application. Aliquots were then mixed with the appropriate volume of Premium Lecithin Organogel Base (Transderma Pharmaceuticals Inc., Coquitlam, BC, Canada) and topically applied onto the shaved dorsal skin of 49-day-old telogen-stage mice. Two experiment time points were used: acute treatment (two doses across 48 hours) or long-term studies (one dose three times a week for approximately 1.5 weeks). Full-thickness dorsal skin was collected for histological analysis, cryosectioning, RNA isolation, or epidermal stem cell isolation according to well-established FACS protocol.

Histology and immunostaining
A set of full-thickness skin samples were obtained from post-mortem tissue harvesting, fixed overnight in 4% paraformaldehyde, and then dehydrated for paraffin embedding and slide generation; hematoxylin and eosin staining was performed according to standard protocol. For immunohistochemistry, formalin-fixed, paraffin-embedded tissue slides were cleared and rehydrated through a series of ethanol washes. Antigen retrieval (20 minutes in pressure cooker, microwaved at 100 power) was performed with 10 mM citrate. Slides were incubated in hydrogen peroxide (30 minutes at 4°C) and then blocked with 10% goat serum/0.1% Tween for 1 hour at room temperature. Primary antibodies were added and incubated overnight. The following antibodies were used: rabbit Sox9 1:800 (Abcam, Cambridge, MA; 185667), rabbit Ki67 1:200 (Abcam 16667), phospho-EGFR.

Figure 2. Topical electron transport chain (ETC) inhibition increases lactate production. (a) Mice were treated topically with the indicated ETC inhibitor for 48 hours. Total epidermis was isolated, lysed, and subjected to lactose dehydrogenase (LDH) activity assay. Relative LDH activity is presented as the rate of activity over 30 minutes in two different animals. (b) Mice were treated topically with ETC inhibitor for 48 hours (top) or 10 days (bottom). Total epidermis was isolated and metabolites were extracted and subjected to metabolomics. Heatmap indicates relative levels of metabolites related to glycolysis and the tricarboxylic acid (TCA) cycle.
1:500 (Abcam 40815), IL-6 1:500 (Abcam 6672), and CD11b 1:250 (550282; BD Pharmingen, San Jose, CA). Sections were washed the following day with 0.1% phosphate buffered saline with Tween and incubated with rabbit secondary horse-radish peroxidase–labeled polymer (Dako, Carpinteria, CA) for 1 hour at room temperature, and then quickly washed with 0.1% phosphate buffered saline with Tween and phosphate buffered saline. AEC chromogen (Dako) was used for the colometric development reaction. Slides were then briefly counterstained with hematoxylin, mounted with Faramount Aqueous Mounting Media (Dako), and sealed for subsequent visualization by light microscopy.

Hair cycle analysis
Hair cycles were assessed macroscopically by photographic documentation. A scale was established to grade hair cycle stage: 1) telogen (pink, white skin when dorsally shaved); 2) pigmentation (brown blue/gray pigment spots/patches on shaved dorsal area); 3) hair growth (dark, full patches of fur); and 4) anagen (dark, full patches of fur within dorsal area) in relation to number of doses and elapsed time of treatment. Hematoxylin and eosin–stained images were captured using 50 magnification to assess morphological changes in control and treated skin. Hair cycle stages were also evaluated by follicular morphology into respective telogen, telogen-to-anagen transition, or anagen categories. Additionally, the epidermis, dermis, and subcutaneous fat layers were independently measured via Imagem software (National Institutes of Health, Bethesda, MD), with approximately 25 measurements/skin layer/animal. Scale was set at 1.68 micrometer/pixel for global layer measurements.

Lactate dehydrogenase plate reader
Lactate dehydrogenase was obtained from cell pellets and resuspended in RIPA buffer with Halt protease and phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA). Staining solutions for lactate dehydrogenase were prepared with Tris buffer (pH 7.4), XTT, nicotinamide-adenine dinucleotide, phenazine methosulfate, substrate (lactate), and reagent-grade water and held at 37°C until use. Solution was added directly onto samples prepared in triplicate across a 96-well plate. A microplate reader held at 37°C measured 457-nm absorbances every 3 minutes across a 3-minute period to assess enzyme kinetics.

Metabolomics
The experiments were performed as described in Flores et al. (2017). Cells were washed with cold 150 mM ammonium acetate (pH 7.3), 1 ml cold 80% MeOH was added, and 10 nmol D/L-norvaline was added. After mixing and pelleting centrifugation, the supernatant was moved to glass vials, dessicated under vacuum, and resuspended in 70% acetonitrile. Five micrometers of sample were injected onto a Luna 3000SRLC (Thermo Scientific) coupled to a Q Exactive mass spectrometer (Thermo Scientific). The Q Exactive run with polarity switching (+3.50 kV/−3.50 kV) in full scan mode with an m/z range of 65–975. Separation was performed using 5 mM NH4AcO (pH 9.9) and acetonitrile. The gradient ran from 15% to 90% over 18 minutes, followed by an isocratic step for 9 minutes and reversal to the initial 15% for 7 minutes. Metabolites were quantified with TraceFinder 3.3 (Thermo Fisher Scientific) using accurate mass measurements (≤3 parts per million) and retention times.

Microscopy
Bright-field immunohistochemistry, hematoxylin and eosin–stained, and Nuclear Fast Red (Sigma, St Louis, MO)–stained images were captured using an Olympus BX51 light microscope (Olympus, Tokyo, Japan).

Statistical analysis
Data were analyzed and error bars represented standard error of the mean. An unpaired, two-tailed Student t test determined significance, with P < 0.05 considered statistically significant, denoted by asterisks (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

Conflict of interest
The authors state no conflict of interest.

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Matilde Miranda1,2, Heather Christofk1,3, D. Leanne Jones1,2,3, and William E. Lowry1,2,3,*
1Molecular Biology Institute, University of California Los Angeles, Los Angeles, California, USA; 2Department of Molecular Cell and Developmental Biology, University of California Los Angeles, Los Angeles, California, USA; and 3Eli and Edythe Broad Center for Regenerative Medicine, University of California Los Angeles, Los Angeles, California, USA
*Corresponding author e-mail: blowry@ucla.edu

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References
Y Miao et al.  
Location-Specific Variations in AGA Hair Follicle  
Identification of Functional Patterns of Androgenetic Alopecia Using Transcriptome Profiling in Distinct Locations of Hair Follicles  

TO THE EDITOR

Androgenetic alopecia (AGA) is a multifactorial disorder caused by synergy of gene, endocrine, and aging. Current studies on AGA mainly focused on the whole hair follicle or the acknowledged target, dermal papillae (DPs). However, hair follicle stem cells (HFSCs) in the bugle area also exert a great influence on AGA. It has been proven that in AGA, the failure of HFSCs to convert to progenitor cells will lead to dysfunction of the hair follicle (Garza et al., 2011). Nonetheless, the exact roles of the different parts of the hair follicle in AGA etiology remain unclear.

To fully investigate location-specific hair follicle gene expression of AGA patients versus healthy individuals, we separated collected hair follicles into two parts by microdissection: the bugle-containing portion and the DP-containing portion (Figure 1b, and see details in the Supplementary Materials and Methods online). Then we performed a thorough transcriptome profiling of six groups: ABD (AGA, balding area, DP-containing part), ABB (AGA, balding area, bugle-containing part), AND (AGA, nonbalding area, DP-containing part), ANB (AGA, nonbalding area, bugle-containing part), CND (control, nonbalding area, DP-containing part), and CNB (control, nonbalding area, bugle-containing part). This study was reviewed and approved by the research ethics board at Nanfang Hospital, Southern Medical University, Guangzhou, China. All contents were adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all participants.

In our profiling results, genes regulating inflammation, stress, and fibrosis were massively overexpressed in all AGA groups compared with control groups (see Supplementary Table S1 online). Consistently, the top gene ontology terms of biological processes that significantly overlapped among the six pairs were oxidative stress, immune regulation, and tissue structure remodeling (see Supplementary Table S2 online). We also noted that balding groups of AGA had the highest level of inflammation, followed by nonbalding groups (Figure 1d), which agreed with the previous research that found that both vertex and occipital regions were implicated in perifollicular inflammation and fibrosis (Nirmal et al., 2013). These findings may account for thinner and poorer-quality occipital hair follicles as AGA progresses, which results in suboptimal hair growth after transplantation. Although the microenvironment and hair follicles in the occipital region are different from balding vertex, the inflammation-stress-fibrosis phenomenon is a common step in occipital and vertex hair follicles.

It has long been recognized that microinflammation is responsible for AGA. Massive infiltration of activated T and B lymphocytes are also found around the upper hair follicle (Mahé et al., 2000). In our transcriptome, key regulators in inflammation, such as AP-1 subunits (FOS, FOSB, JUN, JUNB, etc.), TLRs, PTGS, EGRs, AREG, and HSPA1B were greatly up-regulated in AGA groups. These results were further validated by quantitative PCR and Western blot (Figure 2a–e). Among these inflammation regulators, the bugle-containing parts had more elevated inflammation-related genes than DP-containing parts (Figure 1d). This may be explained by the immune privilege maintained in DP. In contrast, bugle area naturally harbors more antigen-presenting cells equipped to fight off microbial invasion and allergens and is thus a primary target for inflammation. Recent work has proven the vital influence of regulatory T cells on HFSC maintenance (Ali et al., 2017) and that immunocytes are indispensable for hair regrowth (Wang et al., 2017). These findings emphasized the association between inflammation and alopecia.

Inflammatory circumstance is also suspected to disturb HFSC...