

Interplay of MicroRNA-21 and SATB1 in Epidermal Keratinocytes during Skin Aging

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TO THE EDITOR

Aging is a complex process characterized by progressive decline in physiological and biochemical performance of individual tissues and organs. In aged skin, reduced cell proliferation and functional decline of epithelial and mesenchymal cells underlie age-related changes, such as dry skin (xerosis), loss of elasticity, and functional senescence, leading to increased susceptibility to aging-associated conditions such as skin cancer and poor wound healing (Engelke et al., 1997; Zhang et al., 2009). MicroRNAs (miRNAs) are small noncoding RNAs involved in the post-transcriptional regulation of coding-gene expression. They provide an additional level of control for important cellular processes such as growth, differentiation, and remodeling of skin (Botchkareva, 2017). In addition, miRNAs can regulate the expression of important epigenetic regulators, including DNA methyltransferases, histone deacetylases, and polycomb group genes. Disruption of the miRNA-epigenetic regulatory network was shown to interfere with normal physiological cellular functions, leading to activation of disease processes (reviewed in [Sato et al., 2011]). By fine-tuning biological systems, miRNAs can contribute to healthy aging or development of age-related diseases, and may serve as useful diagnostic or prognostic biomarkers for age-related diseases (Olivieri et al., 2012).

MicroRNA-21 (miR-21) plays an important role in the development of a number of pathological skin conditions including psoriasis, tumorigenesis, and poor wound-healing (Ahmed et al., 2011; Meisgen et al., 2012; Yang

et al., 2011). However, the role of miR-21 in skin aging has not been investigated yet. Here, we identify miR-21 as a contributor to skin aging, at least in part, by negative regulation of the chromatin remodeler SATB1 in keratinocytes.

The expression of miR-21 was examined in skin of young and aged mice (8-week-old vs 2-year-old) and human female donors (48, 60–62, and 78 years old; Supplementary Materials and Methods). Quantitative reverse transcriptase-PCR (RT-qPCR) analysis revealed a prominent increase in miR-21 expression in both mouse and human aged skin (Figure 1a and b). In contrast to miR-21, the level of miRNA-199a, which is not detectable in the epidermis of either mouse or human skin, was used as a control for this study (Sonkoly et al., 2007; Yi et al., 2006) and was not altered during the aging process in human skin (Figure 1b). Using in situ hybridization, we confirmed increased miR-21 expression in the epidermis and dermis of aged mouse and human skin (Figure 1c and d). Our data are consistent with published reports showing the elevation of miR-21 in age-associated cardiovascular diseases in human patients (Olivieri et al., 2012) and in kidneys of aged mice (Sataranatarajan et al., 2012).

Human skin aging is associated with a decrease in the expression of keratinocyte differentiation-associated markers (Engelke et al., 1997). Indeed, a significant reduction in the expression of keratinocyte differentiation-related genes was observed in aged mouse ($P < 0.05$) and human skin (Figure 1e and f). Forced expression of miR-21 in both primary mouse and

human keratinocytes transfected with pro-miR-21 mimic resulted in significant reduction in *Krt1* and *Ivl* ($P < 0.05$, mouse) and *KRT1* ($P < 0.01$), *KRT10*, *KRT14*, and *IVL* expressions ($P < 0.05$, human) (Figure 1g and h). This suggests that miR-21 potentially can contribute to skin aging by down-regulating keratinocyte differentiation-related genes, possibly leading to cellular senescence (Dellago et al., 2013) and contributing to increased susceptibility to age-related pathological conditions.

To identify potential putative miR-21 targets, we performed bioinformatics analysis as done previously (Ahmed et al., 2014). By interrogating predicted miR-21 targets from three different databases, we identified 35 potential genes whose expression may be regulated by miR-21. Ten of these genes, including *Satb1*, have highly conserved miR-21 target sequences between human and mouse genomes (Figure 1i). SATB1 is a nuclear protein operating as a genome organizer, which originally was identified as an essential mediator of normal T-cell development regulating the large-scale chromatin remodeling and enhancer–promoter interactions in several lineage-specific gene loci (Cai et al., 2003). In the skin, SATB1 is essential for higher-order chromatin folding and transcriptional regulation of the epidermal differentiation complex locus in keratinocytes (Fessing et al., 2011). Interestingly, genetic ablation of *Satb1* in mouse skin causes thinning of the epidermis accompanied by downregulation in the expression of terminal differentiation-associated genes (Fessing et al., 2011).

We confirmed the direct regulation of *Satb1* by miR-21 using a luciferase reporter assay. Cotransfection of HaCaT cells with pro-miR-21 mimic and the *Satb1* 3' untranslated region reporter construct caused a significant reduction

Abbreviations: miR-21, microRNA-21; miRNA, microRNA; RT-qPCR, quantitative reverse transcriptase-PCR

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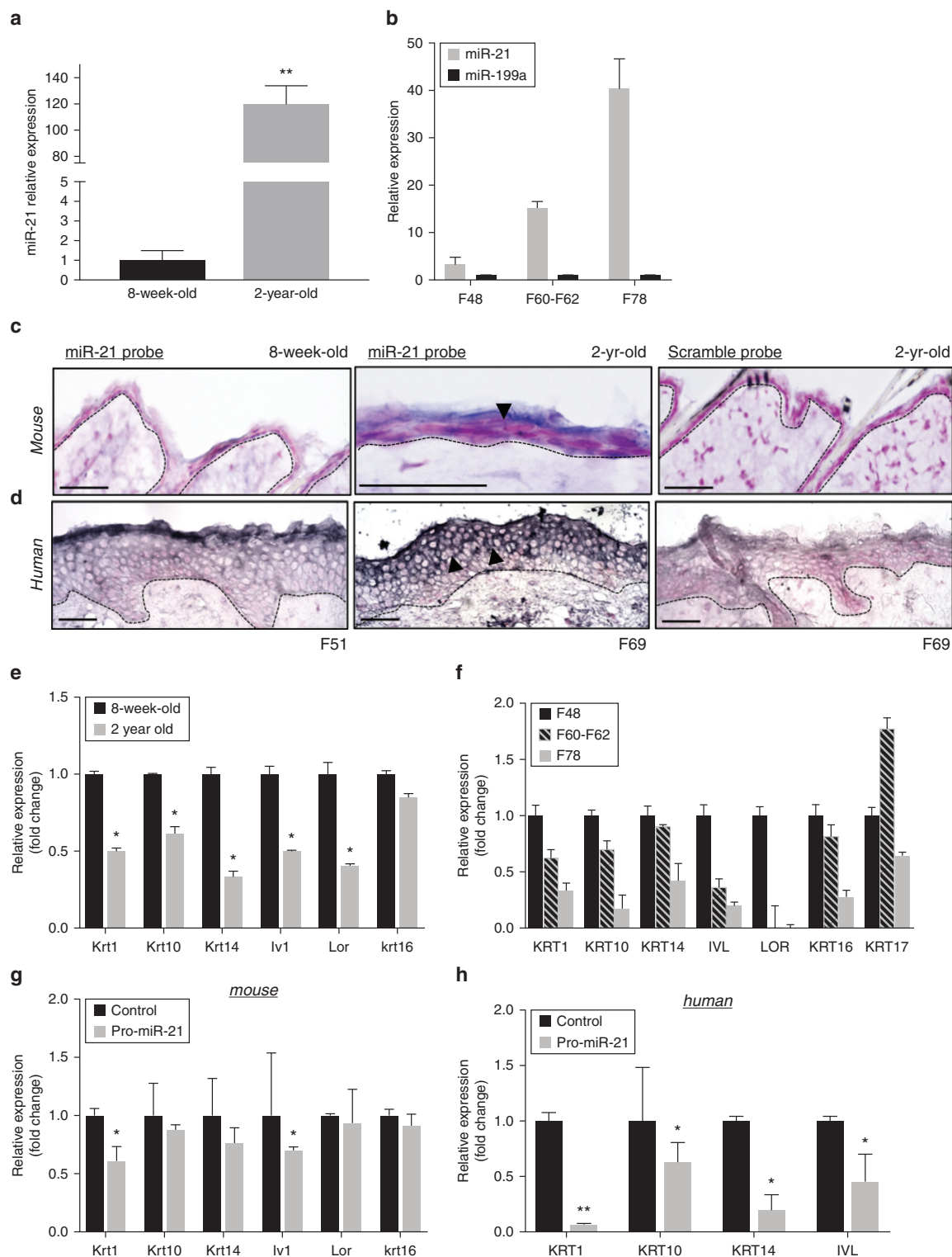


Figure 1. MiR-21 is elevated in aging skin and targets SATB1 in keratinocytes. (a, b) RT-qPCR analysis of miR-21 expression in young vs aged mice (8-week-old vs. 2-year-old) and human female donor skins (F48, F60–62, F78); miR-21 expression is upregulated in aged mouse and human epidermis. Data are presented as mean \pm SEM values from three (mouse) or two (human) independent samples and three independent experiments each. (c, d) Representative photomicrographs of in situ hybridization for miR-21; miR-21 expression is elevated in the epidermis (arrowheads) and dermis of aged mouse and human skins. Data are presented from three (mouse) and two (human) independent samples. (e) RT-qPCR analysis of differentiation-related genes in young versus aged mice; a decrease in expression is observed for all genes analyzed. Data are presented as mean \pm SEM values from three independent samples and experiments. (f) RT-qPCR analysis in young versus aged human skin; a decrease in expression is observed for all differentiation-associated genes analyzed. Data are presented as mean \pm SEM values from two independent samples and three independent experiments. (g, h) Transfection with pro-miR-21 mimic in primary mouse and human keratinocytes causes a significant decrease in the expression of *Krt1* and *Iv1* (mouse) and *KRT1*, *KRT10*, *KRT14*, and *IVL* (human). Data is presented as mean \pm SEM values from three independent experiments. (i) Venn diagram of predicted miR-21 gene targets. A table showing the top ten miR-21 target genes listed as the most conserved between human and mouse genomes, including *SATB1*. (j) Significant reduction in luciferase activity in HaCaT cells cotransfected

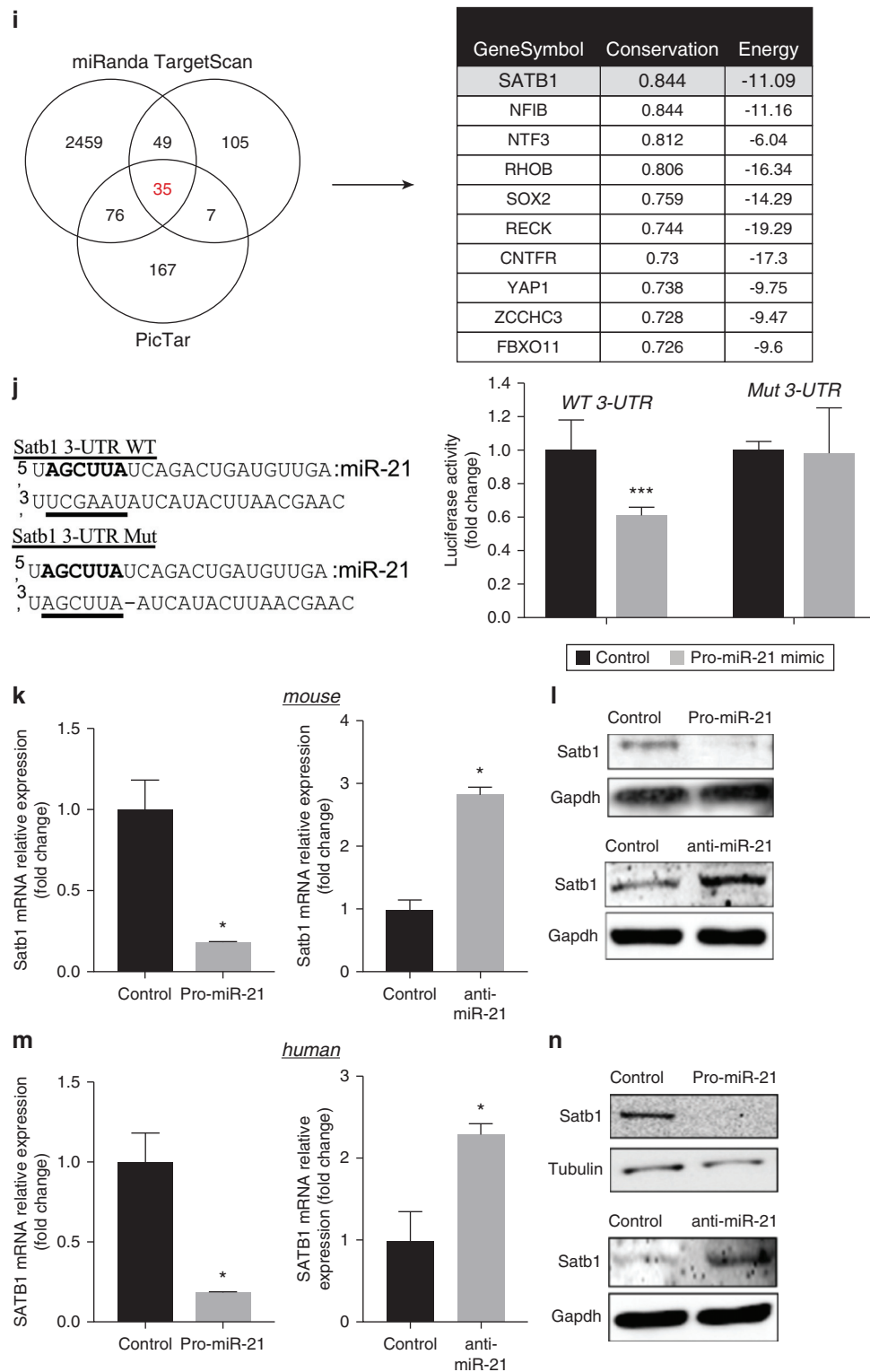


Figure 1. Continued.

with pro-miR-21 mimic and the *SATB1*-3' UTR (wt-3'UTR) construct encompassing the putative miR-21 target site. No changes in luciferase activity were detected when the miRNA binding site was mutated (mut-3'UTR). Bold letters represent miR-21 seed region. Underlined letters represent predicted binding sites within *SATB1*-3'UTR. Each sample was normalized to Renilla luciferase activity. Data is presented as mean \pm SEM values from three independent experiments. (k–n) RT-qPCR and western blot analysis; *SATB1* mRNA and protein levels are significantly decreased and increased after transfection with pro-miR-21 or anti-miR-21, respectively, in both primary mouse and human keratinocytes. Data are presented as mean \pm SEM values from three independent experiments. Western blot data shown are from a single representative experiment out of three repeats. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; Student's *t*-test. The broken lines demarcate the epidermal–dermal border. miR-199a, microRNA-199a; miR-21, microRNA-21; mut, mutated; RT-qPCR, quantitative reverse transcriptase–PCR; SEM, standard error of the mean; UTR, untranslated region; wt, wild type. Bar = 50 μ m.

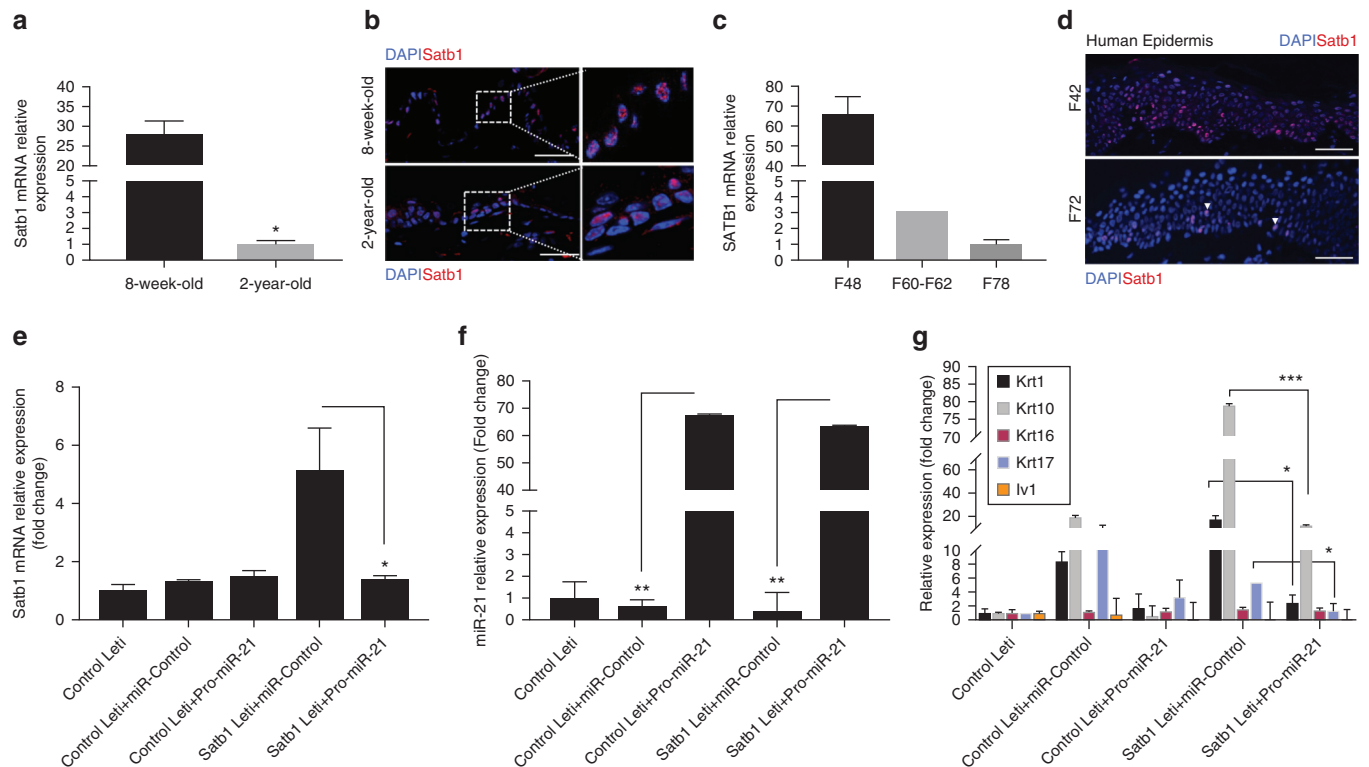


Figure 2. miR-21 modulates expressions of terminal differentiation-associated markers by regulating SATB1 in keratinocytes. SATB1 mRNA and protein expression in (a, b) young versus aged mice (8-week-old vs. 2-year-old) and (c, d) human female donor skins (F48, F60-62, F78). (a, b) SATB1 mRNA and protein expression are downregulated in aged mouse skins. RT-qPCR and immunostaining data are presented as mean \pm SEM values and representative images from three independent samples and experiments. (c, d) SATB1 mRNA and protein expression are downregulated in aged human skin (arrowheads). RT-qPCR and immunostaining are presented as mean \pm SEM values and representative images from two independent samples and three independent experiments. (e, f) *Satb1* and miR-21 expression were upregulated after either *Satb1*-lentiviral overexpression or pro-miR-21 mimic transfection in primary mouse keratinocytes as determined by RT-qPCR analysis. Data are presented as mean \pm SEM values from three independent experiments. (g) RT-qPCR analysis of differentiation-associated genes in primary mouse keratinocytes; overexpression of SATB1 induces expression of *Krt1*, *Krt10*, and *Krt17*, whereas cotransfection with pro-miR-21 mimic diminishes these effects. Data are presented as mean \pm SEM values from three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's *t*-test. miR, microRNA; miR-21, microRNA-21; RT-qPCR, quantitative reverse transcriptase-PCR; SEM, standard error of the mean. Bar = 50 μ m.

in luciferase activity ($P < 0.001$) compared with their corresponding controls, whereas this effect was not detected when miR-21 binding sites in the *Satb1* 3' untranslated region were mutated (Figure 1j). This is consistent with published data showing miR-21 targeting of SATB1 in rectal cancer cells (Lopes-Ramos et al., 2014). The functional interactions of miR-21 and SATB1 in keratinocytes were evaluated by transfecting primary human and mouse epidermal keratinocytes with pro-miR-21 mimic and anti-miR-21, which resulted in the decreased and increased expression of SATB1 mRNA and protein, respectively, as determined by RT-qPCR and western blot (Figure 1k–n, Supplementary Materials and Methods). Additionally, reduced *Satb1* expression was confirmed by RT-qPCR and immunofluorescent analysis

in both mouse and human aged epidermis (Figure 2a–d).

To further explore the plausible functional link between miR-21 and SATB1 in skin aging, we overexpressed SATB1 and miR-21 in keratinocytes using SATB1-expressing lentiviral particles or pro-miR-21 mimic. We confirmed the increased expression of *Satb1* (SATB1 Leti + miR-Control) or miR-21 (Control Leti + pro-miR-21) in primary mouse epidermal keratinocytes as determined by RT-qPCR (Figure 2e and f, Supplementary Materials and Methods). However, coexpression of both SATB1 and miR-21 (SATB1 Leti + pro-miR-21) significantly reduced SATB1 expression (Figure 2e). RT-qPCR analysis also revealed that *Satb1* induces expression of differentiation-associated genes, supporting its role as a promoter of terminal keratinocyte

differentiation (Fessing et al., 2011). Forced expression of miR-21 abolished SATB1-induced upregulation of *Krt1*, *Krt10*, and *Krt17* (Figure 2g). Therefore, our data suggest that miR-21 contributes to the age-associated alterations in gene expression, at least in part, by targeting *Satb1*.

The downregulation of SATB1 in human keratinocytes by miRNA-191 has been shown to establish epigenetic modifications leading to senescence (Lena et al., 2012). SATB1 has also been associated with increased lifespan, whereas a reduction in its expression was seen with age and in age-related pathologies, such as diabetes in mice, demonstrating the general involvement of SATB1 in counteracting the senescence and/or aging pathways (Zhang et al., 2009). An increasing number of studies have identified miR-21 as a

senescence, inflammation, and cancer-associated miRNA (Olivieri et al., 2013). Therefore, our data suggest that the negative regulation of SATB1 by miR-21 in keratinocytes may be an important age-phase-specific regulation leading to senescence and promoting disease states in skin.

Taken together, we demonstrate that (i) miR-21 expression is increased in human and mouse aging skin; (ii) SATB1 expression is inversely correlated with miR-21 in young and aged skin; and (iii) *Satb1* serves as a genuine direct target of miR-21 in keratinocytes. Thus, by regulating SATB1 in epidermal keratinocytes, miR-21 may contribute to the higher-order chromatin remodeling and establishment of enhancer-promoter networks involved in epidermal differentiation, as well as increase susceptibility to age-related pathological conditions, such as tumorigenesis. These data provide a platform for the establishment of novel approaches for pharmacological manipulation of skin aging via modulation of the miR-21 activity in keratinocytes.

ORCID

Mohammed I. Ahmed: <http://orcid.org/0000-0002-9051-7681>
 Maximilian E. Pickup: <http://orcid.org/0000-0002-6006-2540>
 Alexander G. Rimmer: <http://orcid.org/0000-0002-3038-9948>
 Majid Alam: <http://orcid.org/0000-0002-5783-6605>
 Andrei N. Mardaryev: <http://orcid.org/0000-0002-7826-5506>
 Krzysztof Poterłowicz: <http://orcid.org/0000-0001-6173-5674>
 Natalia V. Botchkareva: <http://orcid.org/0000-0002-5202-6822>
 Vladimir A. Botchkarev: <http://orcid.org/0000-0002-9119-0895>

CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: MIA, NVB, VAB; Data Curation: MIA, AGR, KP, MAA, MEP, ANM, NVB, VAB;

Formal Analysis: MIA, AGR, KP, MAA, MEP, ANM, NVB, VAB; Funding Acquisition: MIA, VAB, NVB; Investigation: MIA, AGR, KP, MAA, MEP, ANM, NVB, VAB; Supervision: MIA, NVB, VAB; Writing - Original Draft Preparation: MIA, NVB, VAB.

Mohammed I. Ahmed^{1,*}, Maximilian E. Pickup¹, Alexander G. Rimmer¹, Majid Alam^{2,3,4}, Andrei N. Mardaryev⁵, Krzysztof Poterłowicz⁵, Natalia V. Botchkareva⁵ and Vladimir A. Botchkarev^{5,6}

¹Nottingham Trent University, School of Science and Technology, Nottingham, United Kingdom; ²Mediteknia Skin & Hair Lab, Las Palmas de Gran Canaria, Spain; ³Universidad Fernando Pessoa Canarias, Las Palmas de Gran Canaria, Spain; ⁴Monasterium Laboratory, Muenster, Germany; ⁵Centre for Skin Sciences, Faculty of Life Sciences, University of Bradford, Bradford, United Kingdom; and ⁶Department of Dermatology, Boston University, Boston, Massachusetts

*Corresponding author e-mail: mohammed.ahmed@ntu.ac.uk

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at <https://doi.org/10.1016/j.jid.2019.04.022>.

REFERENCES

- Ahmed MI, Alam M, Emelianov VU, Poterłowicz K, Patel A, Sharov AA, et al. MicroRNA-214 controls skin and hair follicle development by modulating the activity of the Wnt pathway. *J Cell Biol* 2014;207:549–67.
- Ahmed MI, Mardaryev AN, Lewis CJ, Sharov AA, Botchkareva NV. MicroRNA-21 is an important downstream component of BMP signalling in epidermal keratinocytes. *J Cell Sci* 2011;124:3399–404.
- Botchkareva NV. The molecular revolution in cutaneous biology: noncoding RNAs: new molecular players in dermatology and cutaneous biology. *J Invest Dermatol* 2017;137:e105–11.
- Cai S, Han HJ, Kohwi-Shigematsu T. Tissue-specific nuclear architecture and gene expression regulated by SATB1. *Nat Genet* 2003;34:42–51.
- Dellago H, Preschitz-Kammerhofer B, Terlecki-Zaniewicz L, Schreiner C, Fortschegger K, Chang MWF, et al. High levels of oncomiR-21 contribute to the senescence-induced growth arrest in normal human cells and its knock-down increases the replicative lifespan. *Aging Cell* 2013;12:446–58.
- Engelke M, Jensen JM, Ekanayake-Mudiyanselage S, Proksch E. Effects of xerosis and ageing on epidermal proliferation and differentiation. *Br J Dermatol* 1997;137:219–25.

Fessing MY, Mardaryev AN, Gdula MR, Sharov AA, Sharova TY, Rapisarda V, et al. p63 regulates *Satb1* to control tissue-specific chromatin remodeling during development of the epidermis. *J Cell Biol* 2011;194:825–39.

Lena AM, Mancini M, Rivetti di Val Cervo P, Saintigny G, Mahé C, Melino G, et al. MicroRNA-191 triggers keratinocytes senescence by SATB1 and CDK6 downregulation. *Biochem Biophys Res Commun* 2012;423:509–14.

Lopes-Ramos CM, Habr-Gama A, Quevedo Bde S, Felício NM, Bettoni F, Koyama FC, et al. Overexpression of miR-21-5p as a predictive marker for complete tumor regression to neo-adjuvant chemoradiotherapy in rectal cancer patients. *BMC Med Genomics* 2014;7:68.

Meisgen F, Xu N, Wei T, Janson PC, Obad S, Broom O, et al. MiR-21 is up-regulated in psoriasis and suppresses T cell apoptosis. *Exp Dermatol* 2012;21:312–4.

Olivieri F, Rippo MR, Procopio AD, Fazioli F. Circulating inflamma-miRs in aging and age-related diseases. *Front Genet* 2013;4:121.

Olivieri F, Spazzafumo L, Santini G, Lazzarini R, Albertini MC, Rippo MR, et al. Age-related differences in the expression of circulating microRNAs: miR-21 as a new circulating marker of inflammaging. *Mech Ageing Dev* 2012;133:675–85.

Sataranatarajan K, Feliars D, Mariappan MM, Lee HJ, Lee MJ, Day RT, et al. Molecular events in matrix protein metabolism in the aging kidney. *Aging Cell* 2012;11:1065–73.

Sato F, Tsuchiya S, Meltzer SJ, Shimizu K. MicroRNAs and epigenetics. *FEBS Journal* 2011;278:1598–609.

Sonkoly E, Wei T, Janson PCJ, Sääf A, Lundeberg L, Tengvall-Linder M, et al. MicroRNAs: novel regulators involved in the pathogenesis of psoriasis? *PLOS ONE* 2007;2:e610.

Yang X, Wang J, Guo SL, Fan KJ, Li J, Wang YL, et al. miR-21 promotes keratinocyte migration and re-epithelialization during wound healing. *Int J Biol Sci* 2011;7:685–90.

Yi R, O'Carroll D, Pasolli HA, Zhang Z, Dietrich FS, Tarakhovskiy A, et al. Morphogenesis in skin is governed by discrete sets of differentially expressed microRNAs. *Nat Genet* 2006;38:356–62.

Zhang M, Poplawski M, Yen K, Cheng H, Bloss E, Zhu X, et al. Role of CBP and SATB-1 in aging, dietary restriction, and insulin-like signaling. *PLOS Biol* 2009;7:e1000245.



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SUPPLEMENTARY MATERIALS AND METHODS

Animals

Animal studies were performed in accordance with protocols approved by the UK Home Office Project License. C57Bl/6 mice were purchased from Charles River Laboratories. Skin samples were collected at 8 weeks (young sample) and 2 years old (aged samples) and snap frozen in liquid nitrogen for histological and RNA analysis.

Human tissue

Human skin was obtained from healthy donors (42–78 years old) undergoing face-lift and abdominoplasty surgery. Donor age and sites of tissue biopsies can be seen in [Supplementary Table S1](#). Tissue was obtained with full written consent adhering to the Declaration of Helsinki principles, following ethical and institutional approval under human tissue act guidelines.

Quantitative reverse transcriptase–PCR

Quantitative reverse transcriptase–PCR total RNA was isolated using the miR-Neasy Kit (Qiagen, Hilden, Germany). For detection of the mature form of microRNA-21 (miR-21) TaqMan, quantitative reverse transcriptase–PCR was performed using TaqMan Real Time PCR Assay (Applied Biosystems, Foster City, CA) under the following cycling conditions: 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 60 seconds. Differences between samples and controls were calculated based on the Ct ($\Delta\Delta$ Ct) method and normalized to the U6 values. Data from triplicates were pooled, mean \pm standard error of the mean was calculated, and statistical analysis was performed using unpaired Student's *t*-test.

For detection of *Satb1* and epidermal differentiation genes, 1 μ g of total RNA was converted into cDNA using the qPCRBIO cDNA Synthesis Kit system (PCR Biosystems, London, United Kingdom). Gene expression was performed on QuantStudio5 Real Time PCR System (Life Technologies, Carlsbad, CA) using qPCRBIO SyGreen mix (PCR Biosystems). PCR primers were designed with Beacon Designer software (PREMIER Biosoft International, Palo Alto, CA; [Supplementary](#)

[Table S2](#)). Amplification was performed at the following conditions: 95 °C for 5 minutes, followed by 40 cycles of denaturation (95 °C for 15 seconds), annealing (30 seconds at temperature experimentally determined for each primer pairs), and elongation (72 °C for 15 seconds). Differences between samples and controls were calculated based on the Ct ($\Delta\Delta$ Ct) method and normalized to mouse (*Actb*) or human *ACTB* (actin). Data from triplicates were pooled, mean \pm standard error of the mean was calculated, and statistical analysis was performed using unpaired Student's *t*-test.

In situ hybridization

Skin cryosections (10 μ m) were fixed in 4% paraformaldehyde for 10 minutes at room temperature. Tissues sections were acetylated in triethanolamine buffer (4.5 mM triethanolamine, 6 M NCl, and 3 mM acetic anhydride) for 10 minutes and permeabilized (1% Triton X-100/1x diethyl pyrocarbonate–treated phosphate buffered saline) for 30 minutes; slides were hybridized with 2.5 pmol double DIG-labelled miR-21 (Exiqon, Copenhagen, Denmark) and diluted in hybridization buffer (50% formamide DI, 2x saline sodium citrate, 1% dextran sulfate, and 0.4 mg/ml transfer RNA) for 16–18 hours at 60 °C overnight. Slides subsequently were washed in 2x saline sodium citrate (10 minutes, 4 times, 65 °C), 0.1x saline sodium citrate (60 minutes, 65 °C), and 0.2x saline sodium citrate (10 minutes, room temperature). Immunodetection of miR-21 was performed with sheep alkaline phosphatase–conjugated anti-DIG antibody (1:2500, Life Technologies) followed by a staining reaction with BM Purple solution (Life Technologies).

Immunofluorescence

Skin cryosections (10 μ m) were fixed in 4% paraformaldehyde for 10 minutes at room temperature and were stained overnight with rabbit *Satb1* (Abcam, Cambridge, United Kingdom; 1:200, ab49061), followed by application of corresponding donkey anti-rabbit Alexa-555 antibody (Life Technologies, 1:200) for 45 minutes at room temperature. Incubation steps were interspersed by washes with phosphate buffered saline. Sections were counter stained with DAPI.

Cell culture and transfection

Primary human epidermal keratinocytes were isolated as previously described ([Aunin et al., 2017](#)) and grown in keratinocyte media 2 (PromoCell, Heidelberg, Germany), containing 0.06 mM CaCl₂, epidermal growth factor (0.125 ng/ml), and bovine pituitary extract (40 μ g/ml). Primary mouse epidermal keratinocytes were prepared from newborn mice at post-natal days 2–3, as described previously ([Ahmed et al., 2014](#)). Primary mouse epidermal keratinocytes were grown in EMEM calcium-free medium (Lonza, Basel, Switzerland) supplemented with 0.05 mM calcium, at 33 °C, 8% CO₂ (Scientific Laboratory Suppliers, Nottingham, United Kingdom) until 60–70% confluent. Human and mouse keratinocytes were transfected with 200 nM of pro-miR-21 mimic, anti-miR-21, and corresponding miR-controls (Dharmacon, Lafayette, CO) using Lipofectamine RNAiMAX (Life Technologies) as published previously ([Ahmed et al., 2011](#)). Cells were harvested 24 hours (pro-miR-21) and 48 hours (anti-miR-21) after transfection and used for further analyses.

Western blot

Proteins were extracted from cultured cells using RIPA lysis buffer (50 mM Tris-HCl, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, and 1 mM EDTA; pH 7.4) and cComplete ULTRA Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO), as described previously ([Ahmed et al., 2014](#)). In brief, 10–20 micrograms of protein were processed for western blot analysis, followed by membrane incubation with primary antibody against SATB1 (Abcam; ab49061, 1:1,000), glucose-6-phosphate dehydrogenase (Abcam; ab8245, 1:10,000), or tubulin (Abcam; ab7291, 1:20,000) overnight at 4 °C. Horseradish peroxidase–tagged IgG antibodies were used as secondary antibodies (Thermo Fisher Scientific, Waltham, MA; 1:5,000). Antibody binding was visualized with an enhanced chemiluminescence's system (SuperSignal West Pico Kit, Thermo Fisher Scientific) and autoradiographed with X-ray film (CL-Xposure Film, Thermo Fisher Scientific) or iBright Gel-Doc Imager (Thermo Fisher Scientific).

MiR-21 target gene prediction

Possible miR-21 target genes were estimated as a consensus from the following three different prediction algorithms: TargetScan (<http://www.targetscan.org/>) predicts biological targets of microRNAs by searching for the presence of conserved sites that match the seed region of each microRNA, miRanda (<http://microrna.sanger.ac.uk>) uses an algorithm to predict microRNA–mRNA pairs, and PicTar (http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html) confirms candidates predicted by the other two algorithms.

Luciferase reporter assay

HaCaT cells were grown in DMEM (Life Technologies) supplemented with heat-inactivated 10% fetal bovine serum in an atmosphere of 5% CO₂ at 37 °C, until 60–70% confluent as described previously (Ahmed et al., 2014). 3' UTR fragments of *Satb1* containing miR-21 putative target sites were amplified from mouse genomic DNA using forward and reverse primers containing XhoI and NotI restriction sequences, respectively. For 3' UTR of *Satb1* fragment, 5'- CTCCTGCAGCATCATGTCAT -3' and 5'- ACCACTCCTAATCAGCA-CATTTT -3' forward and reverse primers, respectively, were used. Site-directed

mutagenesis was performed using a QuikChange II XL Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA) to mutate the *Satb1* binding site according to the manufacturer's instructions. For the dual luciferase assay, these constructs (200 ng) were cotransfected with 200 nM pro-miR-21 mimic or negative control mimic (Life Technologies) into HaCaT cells using 0.5 µl Lipofectamine 2000 (Life Technologies) in 96-well plates. At 24 hours after transfection, the relative luciferase activities were determined using Dual-Glo Luciferase Assay System (Promega, Madison, WI). The assay was performed in triplicate for three independent trials.

Production of Satb1-expressing virus

For production of control and *Satb1*-expressing lentiviruses, HEK293T cells were cotransfected with control plasmid (Genecopoeia, Rockville, MD) and pEZ-Lv215-*Satb1*-eGFP (Genecopoeia, LPP-Mm19720-Lv215) and helper plasmids (pTAT, pREV, pHagp2 [GAG/Pol], and pVSV-G) using LentiPac HIV Expression Packaging Kit (Genecopoeia, HPK-LvTR) as per the manufacturer's protocol.

Cell culture medium containing viruses was collected 24 hours, 48 hours, and 72 hours after transfection,

followed by precipitation of the viral particles using PEG-it Virus Precipitation Solution (System Biosciences) as per the manufacturer's protocol.

Primary mouse epidermal keratinocytes were infected with *Satb1* and corresponding control lentiviral particles in combination with 10 µg/ml polybrene (Sigma-Aldrich) for 48 hours. To examine the regulatory effects of miR-21 on *Satb1*-induced gene expression, keratinocytes were transfected with 200 nM synthetic pro-miR-21 mimic or microRNA negative controls for 4 hours (post-48-hour lentiviral treatments). All experiment groups were collected for quantitative reverse transcriptase–PCR analysis 24 hours after pro-miR-21 mimic treatment.

REFERENCES

- Ahmed MI, Alam M, Emelianov VU, Poterlowicz K, Patel A, Sharov AA, et al. MicroRNA-214 controls skin and hair follicle development by modulating the activity of the Wnt pathway. *J Cell Biol* 2014;207:549–67.
- Ahmed MI, Mardaryev AN, Lewis CJ, Sharov AA, Botchkareva NV. MicroRNA-21 is an important downstream component of BMP signalling in epidermal keratinocytes. *J Cell Sci* 2011;124:3399–404.
- Aunin E, Broadley D, Ahmed MI, Mardaryev AN, Botchkareva NV. Exploring a Role for Regulatory miRNAs in Wound Healing during Ageing: involvement of miR-200c in wound repair. *Sci Rep* 2017;7:3257.