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 1a and b). 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...
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 ± 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 ± 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 ± 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 Ivl (mouse) and KRT1, KRT10, KRT14, and IVL (human). Data is presented as mean ± 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
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 ± 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 ± 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 µm.

Figure 1. Continued.
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 as a 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
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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 (qPCR) total RNA was isolated using the mirNeasy Kit (Qiagen, Hilden, Germany). For detection of the mature form of microRNA-21 (miR-21) TaqMan, quantitative reverse transcriptase—PCR (qRT-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 (ΔΔCt) method and normalized to the U6 values. Data from triplicates were pooled, mean ± standard error of the mean was calculated, and statistical analysis was performed using unpaired Student’s t-test.

In situ hybridization
Skin cryosections (10 μm) were fixed in 4% paraformaldehyde for 10 minutes at room temperature. Tissue 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).

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 μg/ml). Primary mouse epidermal keratinocytes were prepared from newborn mice at postnatal 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 Supplies, 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) using Lipectamine RNAiMAX (Life Technologies) 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).

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| 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 pair), and elongation (72°C for 15 seconds). Differences between samples and controls were calculated based on the Ct (ΔΔCt) method and normalized to mouse Actb or human ACTB (actin). Data from triplicates were pooled, mean ± standard error of the mean was calculated, and statistical analysis was performed using unpaired Student’s t-test.

Immunofluorescence
Skin cryosections (10 μm) were fixed in 4% paraformaldehyde for 10 minutes at room temperature and were stained overnight with rabbit Satb1 (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’- CTCCGTGACGATCATGTGAT -3' and 5’- ACCACTCTAATCAGCATCATTCC -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 Lenti-Pac 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.

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