Original Article

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The expression of miR-124 increases in aged skin to cause cell senescence and it decreases in squamous cell carcinoma

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Summary **Skin senescence is induced by various factors including intrinsic aging and extrinsic aging. The current study compared the expression of microRNAs in young facial skin and senescent facial skin, and this study identified skin aging-related microRNAs. According to the results from a microRNA PCR Array, miR-124 was the microRNA that increased the most in senescent skin compared to young skin. Real-time PCR with a greater number of samples indicated that the increase in miR-124 levels in senescent facial skin was statistically significant. In situ hybridization was performed, and results indicated that the signal for miR-124 was evident in keratinocytes of senescent skin but not in those of young skin. The morphology of cultured normal human epidermal keratinocytes (NHEKs) transfected with a miR-124 mimic changed to an enlarged and irregular shape. In addition, the number of NHEKs positive for senescence-associated β-galactosidase (SA-β-gal) increased significantly as a result of the overexpression of the miR-124 mimic. The expression of miR-124 increased in UVB-irradiated NHEKs compared to controls in a dose-dependent manner. Expression of miR-124 in A431, a human cutaneous squamous cell carcinoma (SCC) cell line, decreased significantly compared to that in NHEKs. Forced overexpression of miR-124 as a result of the transfection of a miR-124 mimic in A431 resulted in the significant suppression of the proportion of cancer cells. The current results indicated that miR-124 increases as a result of cell senescence and that it decreases during tumorigenesis. The effect of supplementation of miR-124 in an SCC cell line suggests that senescence induction therapy with microRNA may be a new therapeutic approach for treatment of SCC.**

Keywords: Skin senescence, microRNA, UVB

1. Introduction

Based on the characteristics of aging of the skin compared to aging of other organs, there appear to be two different types of aging: intrinsic aging and extrinsic aging. Intrinsic aging is caused by decreased cellular functioning of the skin, while extrinsic aging is mainly induced by UV irradiation. Other factors that affect skin senescence include smoking, eating habits, stress, and oxygen radicals generated by air pollution, but the mediator of these various factors is still unknown.

The current study focused on the role of microRNAs (miRNAs) in skin senescence. miRNAs, short ribonucleic acid molecules an average of 22 nucleotides long, are small noncoding RNAs that lead to gene silencing of target mRNAs (*1*). miRNAs have been implicated in the pathogenesis of various human diseases such as cancers and inflammatory disorders. The current authors previously found that the miR-424 level in senile hemangioma, the most common vascular anomaly seen specifically in aged skin, was lower than levels in other vascular anomalies (*2*): Decreased miR-424 expression and increased levels of target molecules (MEK1 and cyclin E1) in endothelial cells of senile hemangioma may cause abnormal cell proliferation in the tumor.

The current study identified miRNAs that were

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dysregulated in senescent skin, and it attempted to clarify the role of miRNAs in skin senescence.

2. Materials and Methods

2.1. *Patient samples*

Skin samples were obtained from the facial skin of 6 elderly individuals (range: 80-100 years of age) and 3 young individuals (range: 0-10 years of age). Non-exposed parts of the skin of 3 young and elderly individuals were also collected. These samples were skin that was routinely discarded as "dog-ears" during skin surgery, and samples were processed immediately after removal. This study was approved by an institutional review board and written informed consent was obtained from patients in accordance with the Declaration of Helsinki.

2.2. *Cell cultures*

Normal human epidermal keratinocytes (NHEKs) were purchased from Lonza (Walkersville, MD) and were cultured in growth medium (KGM-Gold Bullet Kit, Lonza) in a 5% CO_2 incubator at 37°C. A human cutaneous squamous cell carcinoma (SCC) cell line, A431, was obtained from ATCC (Manassas, VA), and cells were cultured in DMEM (Lonza) with 10% fetal bovine serum (Hyclone, Logan, UT) and Antibiotic-Antimycotic (Invitrogen, Carlsbad, CA) in a 5% CO_2 incubator at 37°C.

2.3. *PCR array analysis and real-time PCR analysis of miRNAs*

Small RNAs were extracted from paraffin sections using the miRNeasy FFPE kit and from cultured cells using the RNeasy mini kit (Qiagen, Valencia, CA) as described previously (*2,3*). For the RT^2 Profiler PCR Array (SABiosciences, Frederick, MD), the RNAs were reverse-transcribed into first-strand cDNA using the RT^2 miRNA First Strand Kit (SABiosciences). The cDNA was mixed with RT^2 SYBR Green/ROX qPCR Master Mix, and the mixture was added to the 96-well RT^2 miRNA PCR Array (SABiosciences) that included primer pairs for 88 human miRNAs. PCR was performed on the Takara Thermal Cycler Dice (TP800) (Takara Bio Inc, Shiga, Japan) in accordance with the manufacturer's protocol. The threshold cycle (C_t) for each miRNA was determined using the Thermal Cycler Dice Real Time System ver2.10B. The raw C_t values were normalized using the values for small RNA housekeeping genes.

For quantitative real-time PCR, primers for miR-124 or U6 (Qiagen) and templates were mixed with SYBR Premix Ex TaqII (Takara Bio Inc). DNA was amplified for 50 cycles of denaturation for 5 s at 95°C and annealing for 30 s at 60°C. Transcript levels of miR-124 were normalized to U6 levels.

2.4. *In situ hybridization*

In situ hybridization was performed using a representative sample with 5'-locked digoxigenin-labeled nucleic acid (LNA) probes complementary to human mature miR-124 and a scrambled negative control (Exiqon, Vedbaek, Denmark) (*1,4*). Sections of human skin were deparaffinized and deproteinized with protease K for 5 min. The slides were then washed in 0.2% glycine in PBS and fixed with 4% paraformaldehyde. Hybridization was performed at 57°C overnight followed by blocking with 2% fetal bovine serum and 2% bovine serum albumin in PBST for 1 h. The probe-target complex was immunologically detected with a digoxigenin antibody conjugated to alkaline phosphatase acting on the chromogen nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche Applied Science, Mannheim, Germany). The slides were counterstained using nuclear fast red and examined under a light microscope (OLYMPUS BX50; Tokyo, Japan).

2.5. *Transient transfection*

miRNA mimics were obtained from Qiagen. Lipofectamine RNAiMAX (Invitrogen) was used as the transfection reagent. For reverse transfection, miRNA mimics were mixed with transfection reagent and then added when cells were plated (*5*).

2.6. *Senescence-associated β-galactosidase (SA- β-Gal) activity*

SA-β-Gal activity was examined using the Cellular Senescence Assay Kit (Chemicon International Inc, Billerica, MA). The cells were treated with fixing solution for 15 min at room temperature and then incubated with SA-β-gal Detection Solution at 37°C overnight. Senescent cells with green staining were counted under a light microscope (*6*).

2.7. *Cell count*

Cells were detached from the wells by trypsin treatment and counted using the Coulter® Particle Counter (Beckman Coulter, Fullerton, CA) (*2,7*).

2.8. *Statistical analysis*

Statistical analysis was performed with the Mann-Whitney test for comparison of medians. p values less than 0.05 were considered significant.

3. Results

3.1. *miRNA expression in young and senescent facial skin*

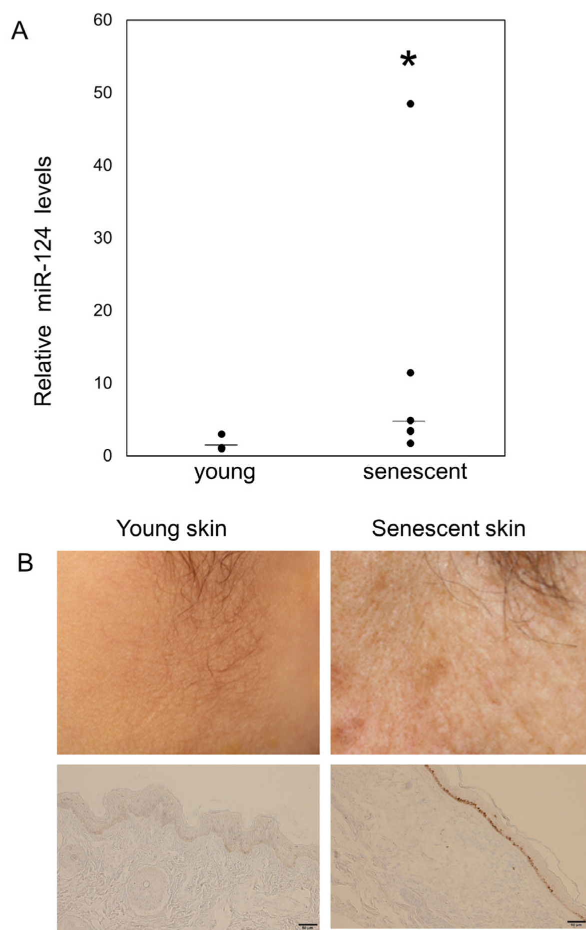

Figure 1. (A) Total miRNA was extracted from samples of young (n = 3) and senescent (n = 6) facial skin. miR-124 levels were measured with real-time PCR and normalized to U6 levels. Relative miR-124 levels are shown on the vertical axis. The minimum value in young skin was set at 1. Bars indicate medians. $p < 0.05$. **(B)** (upper panels) representative pictures of young and senescent facial skin. (lower panels) In situ detection of miR-124 in paraffin-embedded, formalin-fixed tissues from young skin and senescent skin. Nuclei were counterstained with nuclear fast red. The miR-124 stained brown.

Table 1. Up-regulated miRNAs in senescent facial skin compared to young facial skin according to a PCR array

ΔC_t	young	senescent
let-7a	- 0.33	- 3.63
miR-18b	3.80	- 0.29
miR-124	4.09	- 2.43
miR-192	2.30	- 1.37
miR-196a	4.87	- 0.14
miR-206	2.41	- 1.71
miR-208	4.09	0.21
miR-215	4.88	- 1.06
miR-219-5p	6.82	2.07
miR-302c	7.51	3.75
miR-488	5.75	2.00
miR-518b	7.35	2.96

A mixture of equal amounts of miRNAs from facial skin samples from 3 elderly individuals and 3 young individuals were prepared, and the miRNA expression profile in each group in vivo was evaluated using a PCR array. The raw threshold cycle (C_t) was normalized using the values for small RNA housekeeping genes. ΔC_t (the raw C_t for each miRNA – C_t for small RNA housekeeping genes) is shown.

As an initial experiment, to determine which miRNAs are involved in the senescence of facial skin, a mixture of equal amounts of miRNAs from 3 human facial skin samples from young individuals (0-10 years of age) or elderly individuals (80-100 years of age) were prepared (see Figure 1B). A miRNA PCR array was used to analyze 88 miRNAs involved in human cell differentiation and development.

There were several miRNAs that were specifically overexpressed in senescent facial skin (Table 1). The current study focused on one of those miRNAs, miR-124, since it increased the most in senescent skin compared to young skin (6.52-cycle difference in ΔCT method). Because the array was used in a single experiment, the results were verified with real-time PCR using specific primers for miR-124 and a greater number of samples (3 samples of young facial skin and 6 of senescent facial skin). As shown in Figure 1A, the increase in miR-124 levels in senescent facial skin was statistically significant. In addition, in situ hybridization using a representative sample indicated that the signal for miR-124 was evident in keratinocytes of senescent skin but not in those of young skin (Figure 1B).

3.2. *The role of miR-124 in the senescence of keratinocytes*

Next, an attempt was made to determine the role of miR-124 in the senescence of NHEKs. Cells transfected with control miRNA had a cobblestone appearance, while the morphology of cells with overexpression of the miR-124 mimic changed to an enlarged and irregular shape, suggesting cell senescence (Figure 2A). SA-β-Gal staining indicated that the ratio of positive cells increased as a result of the overexpression of the miR-124 mimic compared to that in controls (Figure 2B), and this increase was statistically significant (Figure 2C). Taken together, the current results indicated that miR-124 may be associated with the senescence of keratinocytes.

3.3. *The expression of miR-124 in keratinocytes with UVB irradiation*

Based on above results, the expression of miR-124 was thought to increase in accordance with skin senescence, which means that miR-124 mediates skin senescence. An attempt was made to identify the mechanism by which expression of miR-124 is induced in senescent skin. As mentioned previously, skin senescence is mainly induced by both intrinsic factors and extrinsic factors. UV irradiation is known to be a main cause of extrinsic skin senescence.

The expression of miR-124 increased in UVB-irradiated cells compared to controls in a dose-dependent manner: The induction of miR-124 expression by 10 mJ of irradiation was statistically significant (Figure 3A). In contrast to the results for facial skin, miR-124 levels in non-exposed parts of the skin of elderly individuals

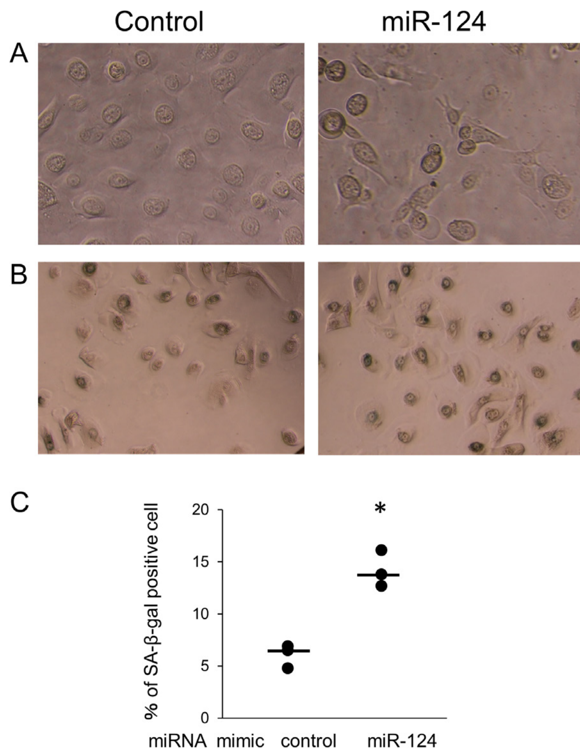

Figure 2. (A) Normal human epidermal keratinocytes (NHEKs) at a density of 6×10^4 cells/well in 24-well culture plates were transfected with a control miRNA mimic or a miR-124 mimic. The morphology of NHEKs was observed using phase-contrast microscopy. **(B, C)** NHEKs at a density of 6×10^4 cells/well in 24-well culture plates were transfected with a control miRNA mimic or an miR-124 mimic. Histochemical detection of SA β-gal in cells was assessed 7 days after transfection **(B)** The percentage of cells positive for SA-β-gal in 3 independent experiments is shown on the vertical axis. Bars indicate medians. $^*p < 0.05$ compared to values in cells transfected with the control mimic **(C)**.

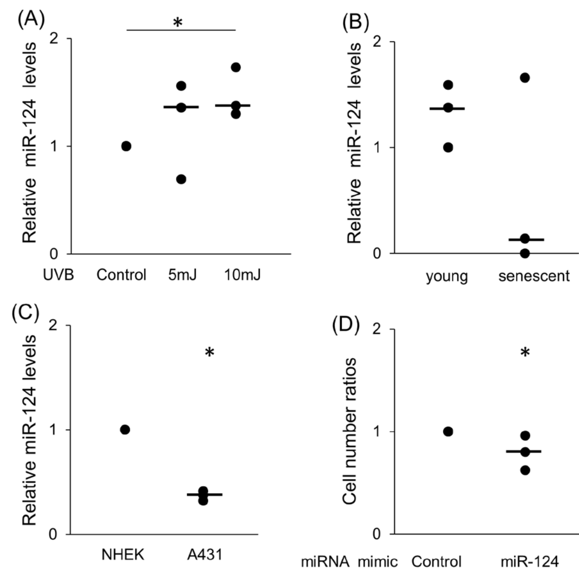

Figure 3. (A) Normal human epidermal keratinocytes (NHEKs) were exposed to UVB at 5 or 10mJ/cm² every day for 3 days. After 24 hours, total miRNA was extracted, and the relative level of miR-124 (normalized to U6) was determined with quantitative real-time PCR (n = 3). Bars show medians. The values in untreated cells were set at 1.0. $^*p < 0.05$ compared to values in untreated cells. **(B)** Total miRNA was extracted from non-exposed parts of skin of young or elderly individuals (n = 3), and the relative level of miR-124 (normalized to U6) was determined using quantitative real-time PCR. Transcript levels in young skin were set at 1. The minimum value in young skin was set at 1. Bars show medians. **(C)** NHEKs and an SCC cell line (A431) were cultured independently under the same conditions until they became subconfluent. Total miRNA was extracted, and the relative level of miR-124 (normalized to U6) was determined using quantitative real-time PCR (n = 3). Bars show medians. $^*p < 0.05$ compared to values in NHEKs (1.0). **(D)** A431 at a density of 5×10^3 cells/well in 24-well culture plates were transfected with a control miRNA mimic or a miR-124 mimic for 72 h. The proportion of cells is shown on the vertical axis (n = 3). Bars show medians. The values in control cells were set at 1.0.

varied and did not increase compared to those in young individuals (Figure 3B). Taking these findings together, miR-124 levels may increase as a result of UV irradiation rather than intrinsic aging.

3.4. *The connection between miR-124 and SCC*

SCC is a malignant skin tumor that is thought to be closely correlated with UVB. The expression of miR-124 in A431, a human cutaneous SCC cell line, decreased significantly compared to that in NHEKs (Figure 3C). Forced overexpression of miR-124 as a result of the transfection of a miR-124 mimic in A431 resulted in the significant suppression of the proportion of cancer cells (Figure 3D). Thus, the current results indicated that miR-124 increased as a result of cell senescence but it decreased during tumorigenesis. Furthermore, the supplementation of miR-124 may suppress tumor proliferation by inducing the senescence of tumor cells.

4. Discussion

To the extent known, no previous studies have examined miRNAs in relation to intrinsic or extrinsic aging. Xu *et al.* indicated that miR-22 plays roles both in cell senescence and tumorigenesis (*8*): the expression of miR-22 is induced by cell senescence while it is reduced in various malignant tumor cells. The authors also indicated that overexpression of miR-22 suppresses the development of breast cancer *in vivo*. The current authors considered the possibility that a similar phenomenon exists in relation to skin senescence. Based on this hypothesis, the current study correlated miRNAs with skin senescence and skin cancer, and this study yielded three major findings.

First, this study attempted to identify skin aging-related miRNAs. Analysis was performed with a miRNA PCR array consisting of 88 miRNAs involved in human cell differentiation and development, and miR-124 was identified as the most up-regulated miRNA in senescent skin compared to young skin. These results were verified by real-time PCR. Attention was then focused on miR-124, and overexpression of miR-124 in NHEKs was found to lead to cell senescence. Although miR-22 was included in the miRNA PCR array, it was similarly

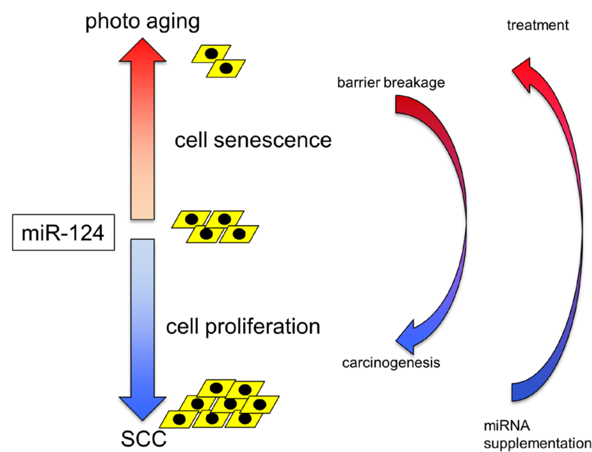

Figure 4. Diagrammatic representation of the function of miR-124 in skin senescence and tumorigenesis. In senescent skin, UVB increases the expression of miR-124, thus mediating cell senescence. In contrast, the expression of miR-124 decreases in SCC tumor cells, and this decrease may correlate with cell proliferation. miR-124 acts as a preventer of skin carcinogenesis, and miR-124 supplementation may have therapeutic value in inhibiting the progression of cancer.

expressed in senescent skin and young skin. This may be due to the tissue specificity of the pattern of miRNA expression.

Second, the expression of miR-124 in NHEKs increased as a result of UVB irradiation in a dose-dependent manner. UVB is a main extrinsic factor for the induction of skin senescence and seems to cause a phenomenon up-stream, resulting in increased miR-124 expression in senescent skin. The connection between UVB and miR-23a, -24, -98, -141 or -365 has previously been reported (*9-13*), but the current results indicated that miR-124 also mediates UVB-induced skin aging.

Third and last, the expression of miR-124 decreased in a human cutaneous SCC cell line in comparison to that in NHEKs, while the overexpression of miR-124 in those cells resulted in the significant suppression of the proportion of cell numbers. The current authors previously reported that the miR-124 expression was down-regulated in cutaneous SCC (*14*), which results in the overexpression of ERK as a target molecule and subsequent cell proliferation. miR-124 was also reported to be involved in the carcinogenesis of various cancers such as glioblastoma, gastric cancer, hepatocellular cancer, breast cancer, and prostate cancer (*15-19*), indicating that miR-124 is a key miRNA in carcinogenesis.

Based on these findings, a hypothetical model was devised as shown in Figure 4. The current results indicated the expression of miR-124 increases in aged skin, causing cell senescence. The effect of supplementation of miR-124 in an SCC cell line suggests that senescence induction therapy with miRNA may be a new therapeutic approach for the treatment of SCC. Limitations of this study are the small sample size, the fact that a single experiment was performed, and the small number of cell lines examined, so further studies are needed in the future. Analysis with the array was performed in a single experiment and statistical analysis could not be performed, but the results were verified using real-time PCR. In addition, in situ hybridization was performed using a single sample of young and senescent skin due to the lack of available samples. Forced miR-124 overexpression in the SCC line reduced the proportion of cells, but this does not mean that decreased expression of miR-124 induces cell proliferation. The association between miR-124 and cell proliferation needs to be clarified in more detail in order to substantiate the hypothesis put forward here.

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