Original Article

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Royal jelly regulates the proliferation of human dermal microvascular endothelial cells through the down-regulation of a photoaging-related microRNA

Yuya Kawano[1], Katsunari Makino[1,*], Masatoshi Jinnin[1,2,*], Soichiro Sawamura[1], Shuichi Shimada[1], Satoshi Fukushima[1], Hironobu Ihn[1]

[1] *Department of Dermatology and Plastic Surgery, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan;*
[2] *Department of Dermatology, Wakayama Medical University Graduate School of Medicine, Kimiidera, Wakayama, Japan.*

Summary **Although royal jelly is believed to prevent skin aging, the underlying mechanism is not known in detail. In the present study, we investigated the plausibility of the involvement of microRNAs in the manifestation of this effect of royal jelly. The expression of microRNAs was determined by PCR array analysis and real-time PCR and the number of cells was counted with a cell counter. Using PCR array, we identified four microRNAs that were downregulated by royal jelly in cultured human dermal microvascular endothelial cells (HDMEC). Upon comparison of the expression of the four microRNAs between young and senescent facial skin, miR-129-5p was found to be significantly upregulated in senescent skin. Consistently, the expression of miR-129-5p in HDMEC was significantly increased by UVB radiation, suggesting that this microRNA is related to photoaging. The royal jelly treatment increased the number of HDMEC. Furthermore, forced overexpression of miR-129-5p resulted in significant decrease in the number of HDMEC, and its forced downregulation increased the number of cells. The number and density of vessels is reported to be decreased in aged skin. Our results indicate that miR-129-5p is induced in damaged endothelial cells upon exposure to UV radiation, which decreases the cell number. Furthermore, administration of royal jelly downregulated the expression of miR-129-5p in endothelial cells, and might prevent skin aging by maintaining the number of cells. The present study elucidates the mechanism of vessel aging caused by UV exposure and the anti-aging effects of royal jelly through the involvement of microRNA.**

Keywords: Endothelial cells, microRNA, photoaging, royal jelly, skin aging

1. Introduction

Skin aging is known to be enhanced by ultraviolet (UV) rays, smoking, stress, and eating habits. These factors affect skin cells, including keratinocytes and fibroblasts, causing epidermal atrophy and denaturation of the extracellular matrix (ECM), which contains collagen and elastic fibers. In addition, aging of endothelial cells has been reported to result in the loss of normal capillaries and in irregular dilation of the remaining vessels (*1-4*). Maintenance of normal vasculature becomes difficult because of these changes, and impaired blood circulation, decreased skin temperature, subcutaneous hematoma (referred to as senile purpura), and delayed wound healing are frequently noticed. The changes in vasculature are thought to play an important role in the problems that occur in aged skin.

Royal jelly is a product synthesized from pollen and is secreted by worker honeybees. It is fed to larvae, and is used in large quantities for the morphological development of queen bees. Although the queen bee and worker bees are genetically identical, the queen's intake of royal jelly prolongs its lifespan by an order-

**Address correspondence to:*
Dr. Katsunari Makino, Department of Dermatology and Plastic Surgery, Faculty of Life Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan.
E-mail: katsuderma@gmail.com

Dr. Masatoshi Jinnin, Department of Dermatology, Wakayama Medical University Graduate School of Medicine, 811-1 Kimiidera, Wakayama, Japan
E-mail: mjin@wakayama-med.ac.jp

of-magnitude over that of worker bees (*5*). In humans, the administration of royal jelly affects various cell types, and its efficacy against aging, hypertension (*6*), and impairment of the motor function (*7*) have been described. Skin aging is also thought to be prevented by the intake of royal jelly (*8*), however, the detailed mechanism for it remains to be elucidated.

In the present study, we sought to determine the role of microRNAs in preventing the skin aging. MicroRNAs are ~22-nucleotide long non-coding RNAs. They regulate gene expression by binding to complementary sequences of 3' untranslated region of their target mRNAs, and thereby, inhibit their translation. Around 2,500 microRNAs have been found in the human genome, which control more than 60% of mRNAs, and are involved in regulating the activity or function of various cell types.

The present study aimed at determining the effect of royal jelly on the expression of microRNAs in skin cells. Furthermore, we elucidated the role microRNAs in the pathogenesis of skin aging and its prevention by the administration of royal jelly.

2. Materials and Methods

2.1. *Reagents*

The royal jelly powder (YDP-M-140830), standardized to contain a minimum of 3.85% (E)-10-hydroxy-2-decenoic acid and 0.67% 10-hydroxydecanoic acid, was obtained from Yamada Bee Co. Inc. (Okayama, Japan). The enzyme-treated royal jelly powder (YRP-M-140906) was standardized to contain a minimum of 3.5% (E)-10-hydroxy-2-decenoic acid and 0.67% 10-hydroxydecanoic acid. The powder was dissolved in distilled water, and used at a dose of 10 μg/mL.

2.2. *Patient samples*

Skin samples were obtained from routinely discarded facial skin of three elderly (aged 80-100 years) and three young (aged 0-15 years) individuals undergoing skin grafting. In the elderly individuals, the collected skin showed manifestations of photoaging, including wrinkles and sagging. Immediately after the removal, the skin samples were fixed in formalin and were embedded in paraffin. The study was approved by the institutional review board, and written informed consent was obtained from the patients in accordance with the Declaration of Helsinki.

2.3. *Cell culture*

Human dermal microvascular endothelial cells (HDMEC) were purchased from Takara (Shiga, Japan), and were cultured in a growth medium (EGM-2MV, Lonza, Walkersville, MD). Normal human epidermal keratinocytes (NHEK) were obtained from Lonza, and were cultured in KGM-Gold (Lonza). Normal human dermal fibroblasts (NHDF) (ATCC, Manassas, VA) were cultured in Minimum Essential Medium (Sigma, St. Louis, MO).

2.4. *PCR array analysis and real-time PCR of microRNAs*

MicroRNAs were extracted from formalin-fixed, paraffin-embedded tissue sections with RNeasy FFPE Kit (QIAGEN, Valencia, CA). Total RNA from cultured cells was extracted using ISOGEN (Nippon Gene, Tokyo, Japan), and microRNAs were separated from total RNA using RT^2 qPCR-Grade miRNA Isolation Kit (SABiosciences, Frederick, MD). For the RT^2 Profiler PCR Array (QIAGEN), RNAs were reverse-transcribed into first-strand cDNAs using the RT^2 miRNA First Strand Kit (QIAGEN). The cDNAs were mixed with RT^2 SYBR Green/ROX qPCR Master Mix, and the mixture was added to the human cell differentiation and development-focused miScript® miRNA PCR Array (96-well format, QIAGEN), which includes primer pairs for 84 human microRNAs. PCR was performed on Takara Thermal Cycler Dice (TP800) (Takara Bio Inc., Shiga, Japan) in accordance with the manufacturer's protocol. The threshold cycle (Ct) for each microRNA was determined using the Thermal Cycler Dice Real Time System Ver.2.10B. The raw Ct values were normalized using the mean of Ct values for small RNA housekeeping genes. The primer sets for U6 (5′-cgcttcacgaatttgcgtg tcat-3′), miR-129-5p (5′-ctttttgcggtctgggcttgc-3′), let-7i-5p (5′-tgaggtagtagtttgtgctgtt-3′), miR-124-3p (5′-taaggcacgcggtgaatgcc-3′), miR-127-5p (5′-ctgaagctcagagggctctgat-3′), and miR-302a-3p (5′-taagtgcttccatgttttggtga-3′) were used in this study.

For quantitative real-time PCR, the primers for miR-129-5p or U6 (QIAGEN) and the templates were mixed with SYBR Premix Ex TaqII (Takara Bio Inc.) (*9*). DNA was amplified for 60 cycles employing a denaturation step of 5 s at 95°C and an annealing step of 20 s at 60°C. The transcript levels of miR-129-5p were normalized to those of U6.

2.5. *Transient transfection*

The mimics and inhibitors of miR-129-5p were obtained from QIAGEN, and Lipofectamine RNAiMAX (Invitrogen) was used as the transfection reagent (*10*). The control mimic and inhibitor were also obtained from QIAGEN. For reverse transfection, microRNA mimics and inhibitors were mixed with the transfection reagent, and then added to the cells at the time of plating.

2.6. *Cell counting*

The cells were detached from the wells by trypsin

Table 1. Expression profiles of microRNAs in cultured cells treated with royal jelly, as measured by PCR array

Items	Endothelial cells			Keratinocytes		Fibroblasts	
	Control	RJ +	RJ –	control	RJ +	control	RJ +
let-7i-5p	1	0.37 ± 0.22*	0.43 ± 0.27*	1	1.84	1	2.89
miR-129-5p	1	0.18 ± 0.18*	0.17 ± 0.18*	1	1.77	1	2.64
miR-302a-3p	1	0.21 ± 0.21*	1.24 ± 1.12	1	93.70	0	0.0039
miR-124-3p	1	60.20 ± 54.95*	24.45 ± 18.79	1	2.14	0	2.87
miR-127-5p	1	0.13 ± 0.10*	1.96 ± 1.62	1	0.37	1	0.58

MicroRNAs were obtained from human dermal microvascular endothelial cells (n = 3), keratinocytes (n = 1), and fibroblasts (n = 1) cultured in the presence or absence of royal jelly (enzymatically treated [RJ+] or untreated [RJ–]) for 24 h. The microRNA expression profile for each cell was determined using the PCR array. The raw threshold cycle (Ct) was normalized using the Ct values for small RNA housekeeping genes. The fold-change was calculated as $1/2^{(\text{raw Ct of each microRNA} - \text{mean Ct of small RNA housekeeping genes})}$. The mean value obtained for the untreated control cells was set as '1'. For endothelial cells, the mean fold-change and standard deviation of each microRNA is shown. * p values < 0.05 compared with the values in control cells.

treatment, and counted using Coulter® Particle Counter (Beckman Coulter, Fullerton, CA), as described previously (*11*).

2.7. *Statistical analysis*

Mann-Whitney test was used for comparison of the median values. The values of means ± SD from three separate experiments are shown in each bar graph. A p value less than 0.05 was considered to be significant.

3. Results

3.1. *Identification of microRNAs regulated by royal jelly in skin cells*

To identify the microRNAs whose expression is regulated by royal jelly, HDMEC (at passages 7, 8, and 9) were cultured in the presence of enzyme-treated (RJ +) or untreated (RJ –) royal jelly for 24 h. For reference, we also added keratinocytes (n = 1) and fibroblasts (n = 1), exposed to enzyme-treated royal jelly, in this array. The expression profiles of 84 microRNAs involved in the differentiation and development of human cells were evaluated in the presence of royal jelly using the PCR array.

The expression levels of five microRNAs were significantly altered (as assessed by Mann-Whitney test) by more than 2-fold in the presence of enzyme-treated royal jelly (Table 1). The expression levels of let-7i-5p and miR-129-5p were significantly reduced by royal jelly that was not treated enzymatically (by 0.43- and 0.17-fold, respectively) as well as by enzyme-treated royal jelly (by 0.37- and 0.18-fold, respectively). Upon enzyme treatment, the proteins present in royal jelly are digested into small peptides, resulting in their better absorption. Given that enzyme-treated and untreated royal jelly suppress the expression of miR-129-5p to the same extent, the down-regulation of miR-129-5p seems to be the main effect of royal jelly. On the other hand, the expression of miR-302a-3p and miR-127-5p was significantly downregulated (by 0.21- and 0.13-fold, respectively) only by the enzyme-treated royal jelly, but not by enzyme-untreated royal jelly (by 1.24- and 1.96-fold, respectively). In addition, the expression of miR-124-3p was only upregulated (by 60.2-fold) by enzyme-treated royal jelly.

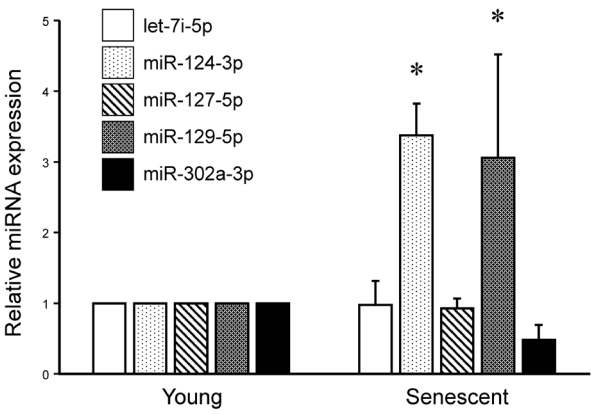

Figure 1. Total microRNA was extracted from skin samples of young (n = 3) and senescent (n = 3) facial skin. The relative expression levels of the five indicated microRNAs (normalized to the expression level of U6) were determined by using quantitative real-time PCR. The values for young skin were set as '1'. Bars indicate the mean values. $p < 0.05$, as assessed by Mann-Whitney test.

3.2. *Expression of miR-129-5p in senescent skin*

The levels of the abovementioned five microRNAs were compared between the facial skin of three young individuals (0-15 years old) and three aging individuals (80-100 years old) (Figure 1). The expression levels of miR-124-3p and miR-129-5p were significantly enhanced ($p < 0.05$) in senescent facial skin. We focused on miR-129-5p because it was the only microRNA whose level was increased in senescent skin by both enzyme-treated and untreated royal jelly. Interestingly, the miR-129-5p levels were not altered by enzyme-treated royal jelly in cultured epidermal keratinocytes or in dermal fibroblasts, as assessed using the array (Table 1). This suggests that miR-129-5p is downregulated by royal jelly specifically in the

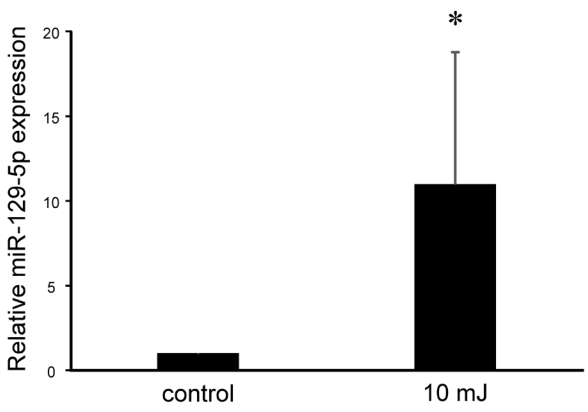

Figure 2. Human dermal microvascular endothelial cells (HDMEC) were exposed to UVB radiation at a dose of 10 mJ/cm²/day for three days. After 24 h, total microRNA was extracted, and the relative level of miR-129-5p (normalized to the expression level of U6) was determined by quantitative real-time PCR (n = 3). Bars show the mean values. *p < 0.05 compared to the values obtained for the untreated cells (1.0).

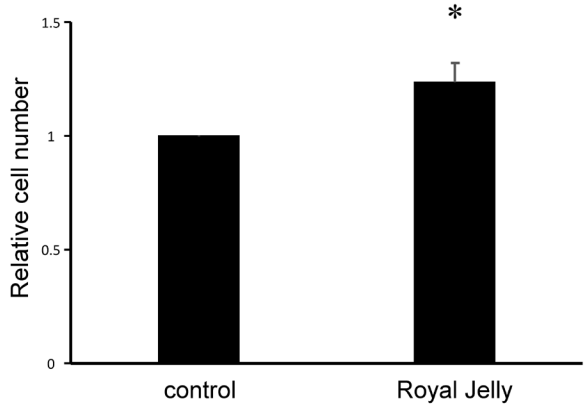

Figure 3. Human dermal microvascular endothelial cells (HDMEC; 1.5 × 10^5 cells/well) were incubated in 6-well culture plates in the presence or absence of enzyme-treated royal jelly (10 µg/mL) for 48 h. The number of cells was counted as described in Materials and Methods section. The value for the untreated cells was set as '1'. *p < 0.05 (n = 3).

endothelial cells. Furthermore, miR-129-5p expression was significantly induced by UV irradiation in HDMEC (Figure 2), indicating that it is the microRNA that is related to photoaging. These findings led us to examine the mechanism through which miR-129-5p affects the function of HDMEC.

3.3. *Significance of reduction in miR-129-5p levels in endothelial cells upon royal jelly treatment*

Next, we determined the function of miR-129-5p in skin aging. Treatment with enzyme-treated royal jelly significantly increased the number of HDMEC (p < 0.05, Figure 3). Royal jelly did not affect the cell viability at 10 µg/mL concentration. On the other hand, the transfection of microRNA mimic specific to

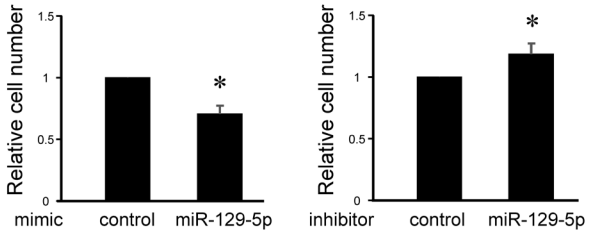

Figure 4. (Left) Human dermal microvascular endothelial cells (HDMEC), at a density of 1.5 × 10^5 cells/well, were transfected in 6-well culture plates with control or miR-129-5p mimic. (Right) HDMEC at a density of 1.5 × 10^5 cells/well were transfected in 6-well culture plates with control or miR-129-5p inhibitor. After 48 h, the number of cells was counted, as described in Materials and Methods section. The values for the cells transfected with the controls were set as '1'. *p < 0.05 compared to the value for the control cells (n = 3).

miR-129-5p significantly decreased the cell number compared to that in the case of control transfection, whereas treatment with the specific miR-129-5p inhibitor increased the cell number (Figure 4).

Based on these results, royal jelly may be thought to induce the cell number of HDMEC through the suppression of miR-129-5p, which is one of the microRNAs related to photoaging.

4. Discussion

Propolis has previously been shown to play a protective role against the arteriosclerosis of vessels by inducing the expression of miR-181a, miR-20b, and miR-106a (*12*). However, to the best of our knowledge, no association has been established between royal jelly and microRNAs, as of date. The present study, which was conducted to test our hypothesis that royal jelly controls skin aging through the regulation of microRNAs, provides three novel insights, as discussed below.

First, as for propolis, herein, we tried to identify microRNAs that are up- or downregulated in human skin cells (endothelial cells, keratinocytes, and fibroblasts), especially in endothelial cells, upon treatment with royal jelly. We performed PCR array of microRNAs involved in the differentiation and development of cells, and identified five microRNAs (let-7i-5p, miR-129-5p, miR-302a-3p, miR-124-3p, and miR-127-5p) that were regulated by royal jelly.

Next, when the expression of five microRNAs was compared between young facial skin and senescent skin, miR-124-3p and miR-129-5p were found to be significantly upregulated in the latter. Consistently, the expression of miR-129-5p in vascular endothelial cells was significantly increased upon exposure to UVB radiation. UVB is one of the main exogenous factors that mediate skin aging. The association between UVB and several microRNAs, such as miR-23a and -23b, was previously reported for other cell types (*13,14*), and our results show, for the first time, that miR-129-5p is also a photoaging-related microRNA in endothelial cells.

Lastly, we evaluated the possibility that royal jelly prevents skin aging through the suppression of miR-129-5p. The number of HDMEC was increased upon addition of royal jelly. Furthermore, the forced overexpression of miR-129-5p resulted in a significant suppression in the number of HDMEC, and forced downregulation of miR-129-5p induced it. It is reported that miR-129-5p negatively controls the proliferation of cells by regulating the cell cycle (*15*). Taken together, our results indicate that miR-129-5p is induced in damaged endothelial cells by UV radiation, which decreases the cell number. Furthermore, the administration of royal jelly downregulated the expression of miR-129-5p in endothelial cells, and may prevent skin aging by maintaining the number of cells. As described in the Introduction section, the number and density of vessels was reported to decrease in aged skin (*1-4*). Herein, we elucidated the mechanism of vessel aging by UV exposure and the anti-aging effects of the royal jelly through the involvement of microRNAs. Also, we previously reported that the expression of miR-124-3p is downregulated in squamous cell carcinoma (SCC), which is one of the most common skin cancers induced by chronic and cumulative UV irradiation (*16*). Because royal jelly enhanced the expression of miR-124-3p in keratinocytes in the present study, it may have preventive effects against SCC.

Further *in vivo* experiments would be required to prove that royal jelly can reduce the expression of miR-129-5p and can prevent photoaging of skin. Furthermore, miR-129-5p might not only be involved in increasing the number of cells, but might also prevent skin aging through the regulation of its target genes. However, further work would be needed to explore these molecular mechanisms. Many genes are predicted as putative targets of miR-129-5p using Targetscan (*http://www.targetscan.org*). These include calcium signaling-related molecules, such as calcium channel, voltage-dependent, γ subunit 2 (CACNG), calcium/calmodulin-dependent protein kinase II inhibitor 1 (CAMK2N1), FK506 binding protein 2, 13 kDa (FKBP2), and calmodulin 1 (phosphorylase kinase, delta) (CALM1). Considering the reported association between skin aging and calcium signaling (*17*), miR-129-5p could be one of the important microRNAs involved in skin aging. In addition, the regulation of calcium signaling by miR-129-5p may cause several changes, such as calcification or arteriosclerosis, in aged vessels.

Acknowledgements

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Conflict of Interest

Royal Jelly, both enzymatically treated and untreated, was provided in powdered form by Yamada Bee Company Inc., Okayama, Japan. The company had no role in the study design, data collection and analysis, decision to publish, or in the preparation of the manuscript. There are no other conflicts of interests to declare.

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