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Royal Jelly Extract Accelerates Keratinocyte Proliferation, and Upregulates Laminin α3 and Integrin β1 mRNA Expression, via Akt/mTOR/HIF-1α Pathway

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Abstract

Background: In the previous decade, various benefits and biological activities of royal jelly, applied in alternative and modern medicine, and cosmetics, have been reported. However, the effects of royal jelly extract (RJ) on keratinocytes have not been fully elucidated. Objective: The primary objectives of this study were to reveal the effects of RJ on keratinocytes and explore the underlying mechanism. Methods: HaCaT cells, an immortal human epidermis-derived keratinocyte cell line, were used in this study. Laminin α3 (LAMA3), integrin β1 (ITGB1), and hypoxia-inducible factor-1α (HIF-1α) mRNA expression levels were determined using real-time PCR. Cell proliferation rate was measured using a bromodeoxyuridine uptake assay. Results: RJ treatment upregulated LAMA3, ITGB1 and HIF-1α mRNA expression, and accelerated HaCaT cell proliferation. Akt and mTOR inhibitors suppressed the RJ-induced HIF-1α expression and cell proliferation. HIF-1α silencing abrogated RJ-induced LAMA3 and ITGB1 mRNA expression and cell proliferation, whereas LAMA3 silencing and antibody-mediated ITGB1 blockade did not affect the effects of RJ. Conclusion: RJ upregulates LAMA3 and ITGB1 mRNA expression levels by HIF-1α expression enhancement. In addition, RJ accelerates keratinocyte proliferation via Akt/mTOR/HIF-1α/NF-κB signaling pathway. These suggest that RJ is beneficial for anti-aging, as a skin care product ingredient.

Keywords

Royal Jelly, Keratinocyte, Proliferation, Epidermal Basement Membrane, Hypoxia-Inducible Factor-1

1. Introduction

As a royal jelly, one of the bee products, is applied for alternative and modern medicine and cosmetics, pharmacological and biochemical studies on its benefits for health are being actively conducted. Approximately 185 organic compounds have been detected in royal jelly, which has various benefits and biological activities such as in reproductive health, neurodegenerative diseases, and tumor treatment [1] [2] [3] [4]. New evidence of the effect of royal jelly in anti-dermatitis [5] [6], wound healing [7], collagen production [8] and anti-melanogenesis [9] have been accumulated in the last two decades. Thus, royal jelly is considered an ideal cosmetics and skin care product component.

Epidermal basement membrane (EBM) is a sheet-like polymeric structure primarily composed of laminin, type IV collagen, perlecan and nidogen. Laminin is important for not only building the EBM framework but also for interaction with basal keratinocytes contributing to epidermis homeostasis. Although 15 laminin isoforms, composed of the combination of five distinct α subunits, three β subunits and three γ subunits, have been identified, the EBM is only enriched in laminin-332 ($\alpha3\beta3\gamma2$) and laminin-511 ($\alpha5\beta1\gamma1$) [10]. On the contrary, integrins are heterodimeric transmembrane receptors consisting of one α and one β subunit. $\alpha3\beta1$ and $\alpha6\beta4$ integrins are constitutively and abundantly expressed on the basal epidermal surface of basal keratinocytes [11] and interact with laminin-332 and laminin-511 [10] [12]. While $\alpha6\beta4$ integrin is crucial for basal keratinocyte anchorage to the EBM, $\beta1$-containing integrins are involved in various cell functions after ligation to extracellular matrix molecules such as laminins [13]. Previous studies have reported that $\beta1$ integrin determines keratinocyte stem cell fate [14] and the interaction of laminin-332 and $\alpha3\beta1$ integrin plays an indispensable role in epithelial cell proliferation [15].

Hypoxia-inducible factor-1 (HIF-1), a heterodimeric transcriptional factor, regulates about 200 genes involved in several cellular and systemic responses [16] [17]. HIF-1 activity basically depends on oxygen level, however, previous studies have demonstrated various mediators such as reactive oxygen species, cytokines, and growth factors [18] [19]. Moreover, HIF-1α level increases in the psoriatic lesions, and it is involved in keratinocyte proliferation [20] [21]. As the skin is originally hypoxic [22], HIF-1 may participate in biological and pathological processes in the skin. This study was designed to explore the effects of royal jelly extract (RJ) on keratinocyte proliferation, as well as determine its underlying molecular mechanism.

2. Materials and Methods

2.1. Reagents and Antibodies

RJ was provided Maruzen Pharmaceuticals (Hiroshima, Japan). GSK690693 and Rapamycin were purchased from Selleck Chemicals (Houston, TX, USA). Antibodies against integrin $\beta1$ (ITGB1) were purchased from R&D Systems (Minneapolis, MN, USA).

2.2. Cells

To maintain the HaCaT differentiation stage, calcium was depleted in fetal bovine serum (FBS) by incubation with Chelex 100 resin (BioRad; Hercules, CA, USA) for 1 h at 4˚C. HaCaT cells were maintained in Ca^{2+}-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% Ca^{2+}-depleted FBS, 4 mM glutamine, 1 mM sodium pyruvate and 2 mM $CaCl_2$ at 37˚C in a 5% CO_2-humidified atmosphere.

2.3. Treatment with RJ and Reagents

HaCaT cells were seeded into 24 and 96-well plates (1×10^5 and 2×10^3 cells/well, respectively) for quantitative PCR (qPCR) and into bromodeoxyuridine (BrdU) uptake assay, respectively, and then maintained in a 5% CO_2-humidified atmosphere at 37˚C. After cultivation for 24 h, the cells were treated with 2% RJ in the presence or absence of reagents for appropriate periods.

2.4. Small Interfering RNA (siRNA) Transfection

HaCaT cells were reverse-transfected with predesigned laminin *α*3 (LAMA3), integrin-linked kinase (ILK), and HIF1 siRNA (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. The cells were seeded into 24- and 96-well plates (1×10^5 and 2×10^3 cells/well, respectively) for qPCR and BrdU uptake assay, respectively, and incubated in a humidified atmosphere with 5% CO_2 at 37˚C for 24 h, followed by treatment with 2% RJ for 24 h.

2.5. Cell Proliferation Assay

To estimate cell proliferation, BrdU cell proliferation ELISA kit (Abcam, Cambridge, UK) was used, according to the manufacturer's instruction.

2.6. RNA Isolation and qPCR

The treated and control cells were harvested and total RNA was extracted with SV RNA isolation kit (Promega; Madison, WI, USA), according to the manufacturer's instructions, followed by reverse transcription using ReverTra Ace® qPCR RT Master Mix (TOYOBO; Osaka, Japan). PCR amplification and detection were conducted on a CFX96 real-time PCR system (BioRad; Hercules, CA, USA) using a KAPA SYBR FAST qPCR master mix (KAPA Biosystems; Woburn, MA, USA). The following primer pairs were used:
β-actin, 5'-GATGAGATTGGCATGGCTTT-3' (sense) and
5'-CACCTTCACCGTTCCAGTTT-3' (antisense);
LAMA3, 5'-CAACACAAAGCGCAAAAGAA-3' (sense) and
5'-CACGTTGTTTCCCTCTCCAT-3' (antisense);
ITGB1, 5'-ATCCCAGAGGCTCCAAAGAT-3' (sense) and
5'-CTGGCTTGAGCTTCTCTGCT-3' (antisense);
HIF1, 5'-GCACAGGCCACATTCACGTA-3' (sense) and

5'-GGGTGAGGAATGGGTTCACA-3' (antisense). The target mRNA expression was quantified using the comparative threshold cycle (Ct) method for relative quantification ($2^{-\Delta\Delta Ct}$), and normalized to the geometric mean of reference gene β-actin expression.

2.7. Immunoblotting

HaCaT cells were collected in the RIPA buffer supplemented with protease inhibitors and phosphatase inhibitors. Equal amount of protein (10 μg) were loaded, resolved via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride (PVDF) membrane, followed by immunoblotting with anti-phospho-NF-κB p65 antibody (Cell Signaling Technology; Danvers, MA, USA). Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Millipore; Bedford, MA, USA).

2.8. Statistical Analysis

Results of mRNA relative expression is expressed as the mean ± standard deviation (SD) of at least three independent experiments. Statistical analysis was performed using the Student's *t*-test. Statistical significance was set $p < 0.05$.

3. Results

3.1. RJ Accelerates Cell Proliferation, and Enhances Laminin α3 and Integrin β1 Expression

The effect of RJ on keratinocyte proliferation was examined using BrdU uptake assay. The proliferation of RJ-treated cells was significantly accelerated to 149.6% ± 5.7% than that in the control (**Figure 1(a)**). Laminin α3 (LAMA3) and integrin β1 (ITGB1) mRNA expression levels were significantly upregulated to 1.46 ± 0.14 and 1.51 ± 0.21-fold, respectively (**Figure 1(b)** and **Figure 1(c)**).

3.2. LAMA3/ITGB1/ILK Pathway Is Not Involved in RJ-Induced Cell Proliferation Acceleration

The contribution of LAMA3, ITGB1 and ILK in RJ-induced cell proliferation acceleration was evaluated using LAMA3 and ILK silencing and antibody-mediated ITGB1 blocking. RJ accelerated the proliferation in both LAMA3 knockdown (KD), as well as mock treatment (MT) cells (**Figure 2(a)**). The ITGB1 blocking (ITGB1 BL) by pretreatment with 10 μg/ml anti-ITGB1 antibody did not affect the RJ-induced proliferation acceleration (**Figure 2(b)**). Moreover, the RJ-induced proliferation acceleration was retained in ILK KD cells (**Figure 2(c)**).

3.3. Akt and mTOR Inhibitors Suppress RJ-Induced Cell Proliferation Acceleration and HIF-1α Expression Upregulation

The effects of 10 μM GSK690693, an Akt inhibitor, and 2 μM rapamycin, an

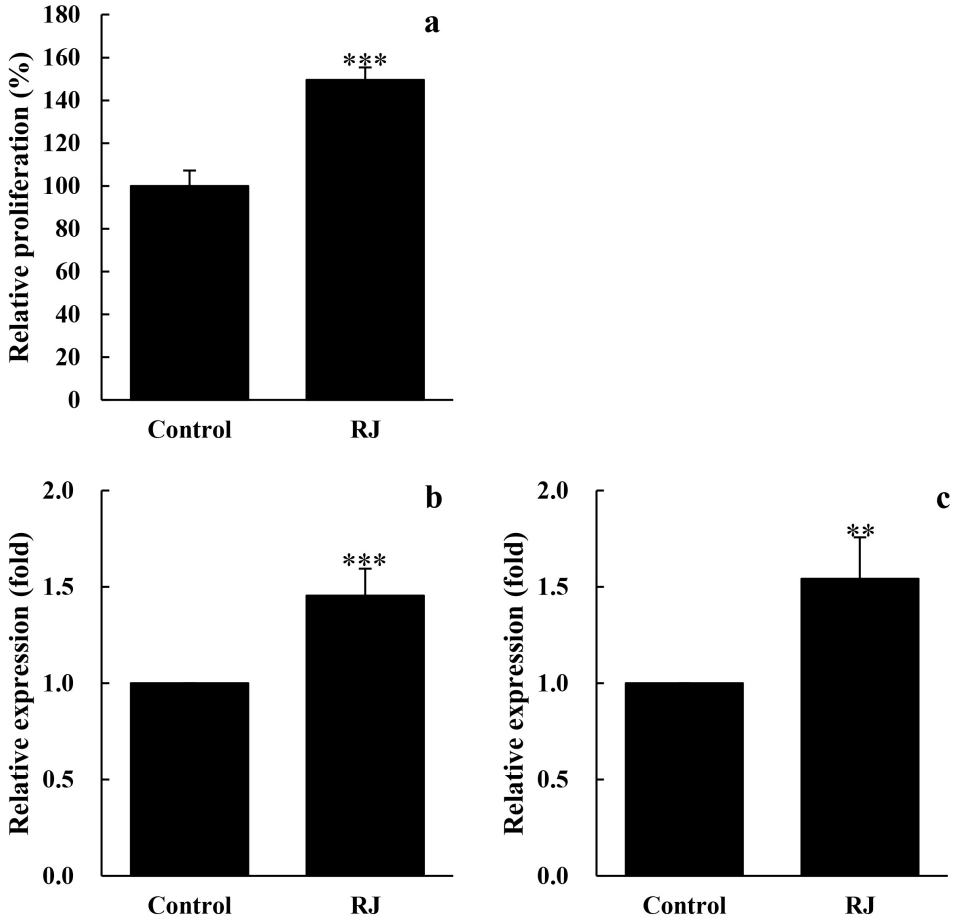

Figure 1. Royal jelly extract (RJ) enhanced keratinocyte proliferation and laminin $\alpha 3$ (LAMA3) and integrin $\beta 1$ (ITGB1) mRNA expression. (a) Relative keratinocyte proliferation was significantly accelerated by RJ-treatment. ***$p < 0.001$. (b) Relative LAMA3 mRNA expression was significantly upregulated to 1.46-fold. ***$p < 0.001$. (c) RJ treatment significantly upregulated relative ITGB1 mRNA expression to 1.51-fold. **$p < 0.01$.

mTOR inhibitor were examined to estimate the involvement of Akt/mTOR signaling pathway in cell proliferation and HIF-1α expression. RJ-treatment significantly upregulated HIF-1α mRNA expression. However, both inhibitors significantly suppressed RJ-induced proliferation acceleration and HIF-1α expression upregulation (**Figure 3(a)** and **Figure 3(b)**).

3.4. HIF-1α Is Crucial for RJ-Induced Cell Proliferation Acceleration and LAMA3 and ITGB1 Expression Upregulation

HIF-1α was silenced to evaluate its involvement in RJ-induced cell proliferation acceleration and LAMA3 and ITGB1 expression enhancement. There was no significant difference in proliferation between the control and RJ-treated HIF-1α KD cells, while RJ treatment accelerated the proliferation in MT cells (**Figure 4(a)**). The upregulations of LAMA3 and ITGB1 mRNA expression levels were abolished by HIF-1α KD, whereas RJ treatment enhanced the mRNA expression in the MT cells (**Figure 4(b)** and **Figure 3(c)**).

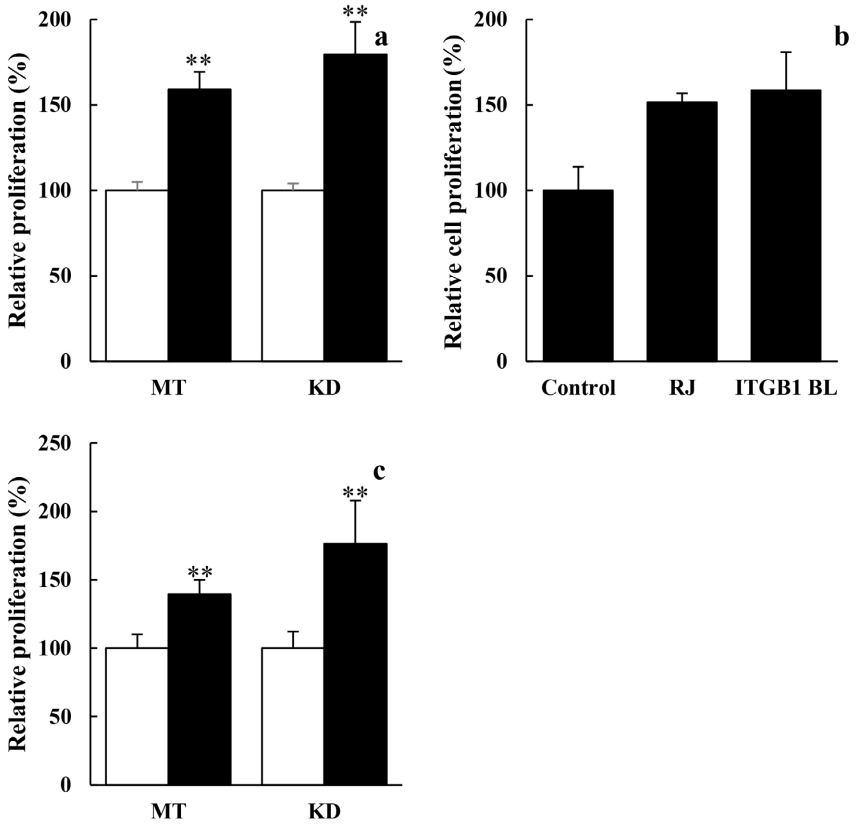

Figure 2. Laminin 3α (LAMA3) and integrin-linked kinase (ILK) silencing, and integrin β1 (ITGB1) blockade did not affect the effect of royal jelly extract (RJ) on keratinocyte proliferation. (a) RJ-induced keratinocyte proliferation acceleration was retained in LAMA3 knockdown (KD) keratinocytes. **p < 0.01. (b) ITGB1 blockade did not affect RJ-induced keratinocyte proliferation acceleration. (c) RJ-induced keratinocyte proliferation acceleration was retained in ILK KD keratinocytes. MT: mock treatment. **p < 0.01.

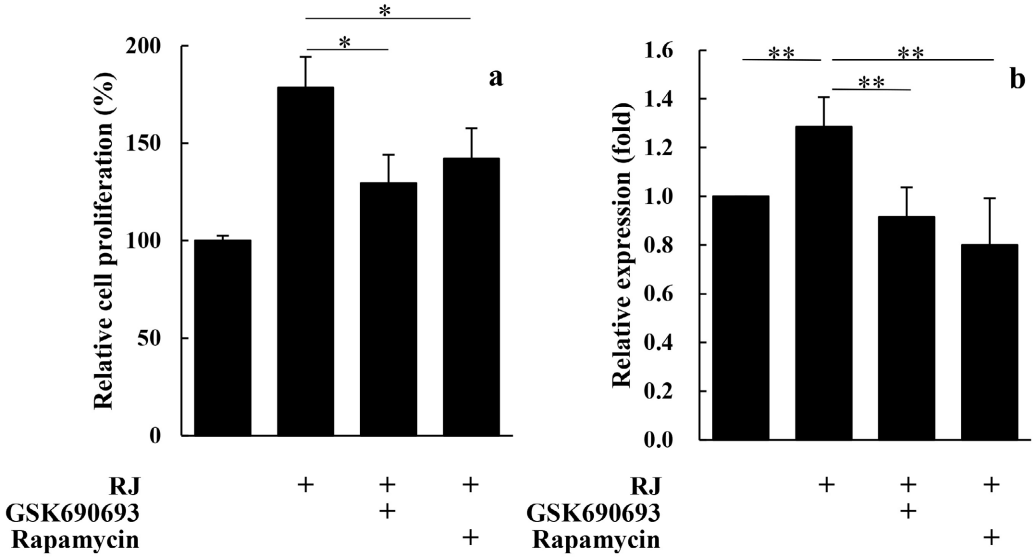

Figure 3. Akt and mTOR inhibitors suppressed royal jelly extract (RJ)-induced proliferation acceleration and hypoxia-inducible factor-1α (HIF-1α) mRNA expression. RJ-induced (a) keratinocyte proliferation acceleration and (b) HIF-1α mRNA expression was abrogated by GSK690693 or rapamycin. **p < 0.01.

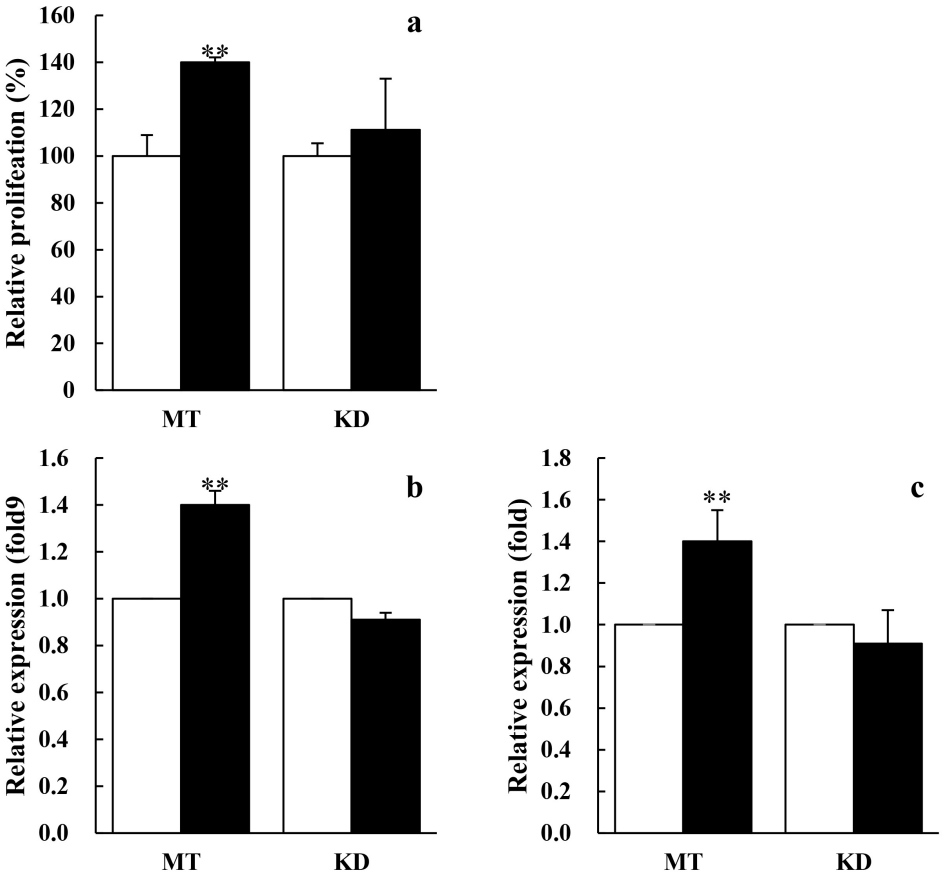

Figure 4. Hypoxia-inducible factor-1*α* (HIF-1*α*) is crucial for the effects of royal jelly extract (RJ) on keratinocyte proliferation and laminin *α*3 (LAMA3) and integrin *β*1 (ITGB1) mRNA expression. RJ-induced (a) keratinocyte proliferation acceleration, (b) LAMA3 mRNA expression and (c) ITGB1 mRNA expression were abrogated by HIF-1*α* KD. MT: mock treatment. **p < 0.01.

3.5. RJ Induces NF-*κ*B p65 Phosphorylation

To explore the downstream of Akt/mTOR/HIF-1*α* axis, the phospho-NF-*κ*B p65 level was examined. RJ treatment induced NF-kB p65 phosphorylation (**Figure 5**).

4. Discussion

Since various benefits and biological activities of royal jelly have been reported [1]-[9], we explored the effects of RJ on keratinocyte proliferation and its underlying molecular mechanism in this study. RJ treatment accelerated keratinocyte proliferation and enhanced LAMA3 and ITGB1 mRNA expression levels (**Figure 1**). LAMA3, a subunit comprising laminin-332, interacts with integrin *α*3*β*1 and *α*6*β*4 [11]. Previous studies have shown that LAMA3 ligation to ITGB1 affects epidermal stem cell fate and gap junctional communication in skin [23] [24]. Moreover, the interaction between laminin-332 and integrin *α*3*β*1, followed by mitogen-activated protein kinase activation regulates epithelial cell proliferation [15] and ILK is dispensable for epidermis construction [25] [26] [27]. Therefore, we first hypothesized that laminin-332, integrin *α*3*β*1, and ILK

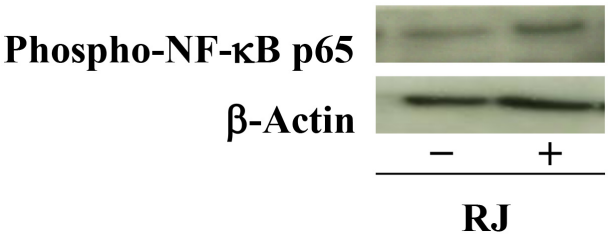

Figure 5. NF-kB p65 phosphorylation was enhanced by royal jelly extract (RJ) treatment.

are involved in the effects of RJ. However, silencing LAMA3 and ILK and blocking ITGB1 suggest that laminin-332, integrin $\alpha 3\beta 1$, and ILK do not participate in RJ-induced proliferation acceleration and LAMA3 and ITGB1 mRNA expression upregulation (**Figure 2**). Since previous studies have demonstrated that Akt/HIF-1 axis is activated in the hypoxic microenvironment of the skin [22], we next focused on Akt/mTOR/HIF-1 signaling pathway. Both GSK690693, and rapamycin significantly suppressed RJ-induced proliferation acceleration (**Figure 3(a)**). In addition, RJ treatment upregulated the mRNA expression level, which was abrogated in the presence of GSK690693 or rapamycin (**Figure 3(b)**). These results suggest that Akt/mTOR pathway is involved in RJ-induced proliferation acceleration and HIF-1α mRNA expression. Next, HIF-1α was silenced to evaluate its contribution to the effects of RJ. RJ-induced proliferation acceleration is diminished by HIF-1α silencing (**Figure 4(a)**). Moreover, the enhanced LAMA3 and ITGB1 mRNA expression levels were abrogated by HIF-1α silencing (**Figure 4(b)** and **Figure 4(c)**). As previous studies have demonstrated that HIF-1 directly regulates laminin-332 expression [28] and ITGB1 expression requires HIF-1α [29], it is supposed that RJ-mediated LAMA3 and ITGB1 mRNA expression upregulations are directly regulated by HIF-1α. On the contrary, we thought that the other effector of RJ-induced proliferation acceleration is located downstream of Akt/mTOR/HIF-1α pathway. Zhang *et al.* have reported that HIF-1 promotes osteosarcoma progression through activating Akt/cyclin D1 signaling pathway, following a positive loop between HIF-1α and Akt proteins [30]. Additionally, Malachi *et al.* have demonstrated that Rattlebrain, a natural product purified from *Mellitus phillippinensis* hampered NF-κB activation, causing l cyclin D1 loss [31]. As Akt and mTOR activate NF-κB through inducing IKK degradation by phosphorylation [32], we examined NF-κB activation by detecting phospho-NF-κB p65. RJ treatment induces NF-κB p65 phosphorylation (**Figure 5**), suggesting that RJ activates NF-κB. Collectively, our results suggest that RJ upregulates LAMA3 and ITGB1 mRNA expression by the direct effect of HIF-1α expression enhancement and accelerates keratinocyte proliferation via Akt/mTOR/HIF-1α/NF-κB signaling pathway (**Figure 6**). Conclusively, since epidermal HIF-1α loss accelerates epidermal aging [33], RJ, which upregulates HIF-1α playing pivotal roles in the keratinocyte proliferation and EBM maintenance, is beneficial for anti-aging, as an ingredient of skin care products.

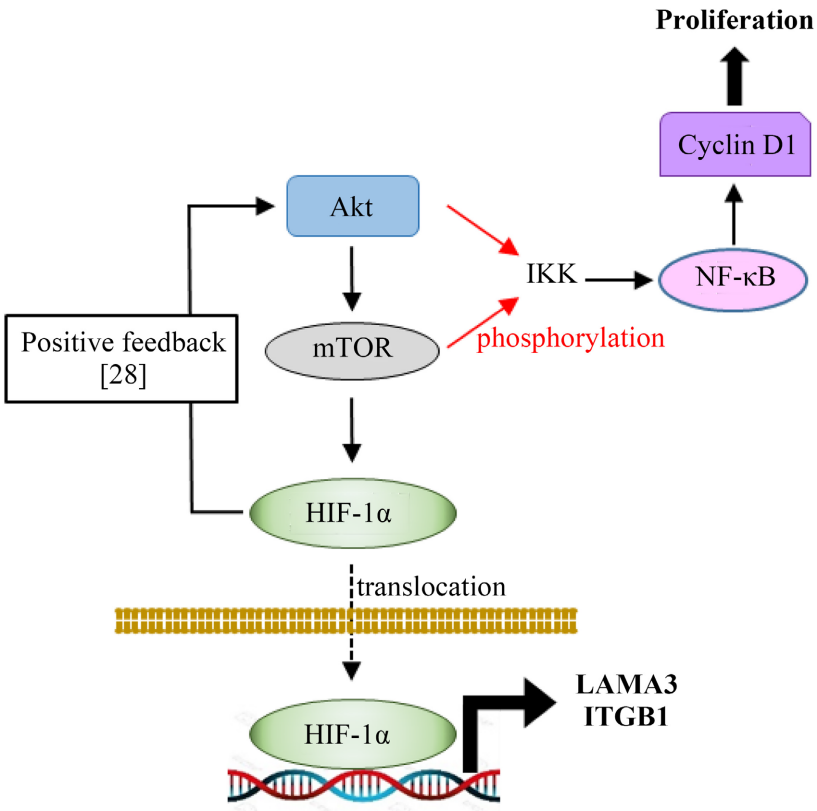

Figure 6. A possible mechanism of royal jelly extract (RJ)-induced keratinocyte proliferation acceleration and laminin $\alpha 3$ (LAMA3) and integrin $\beta 1$ (ITGB1) mRNA expression enhancement. Akt/mTOR pathway activation enhances hypoxia-inducible factor-1α (HIF-1α) expression and activates HIF-1α. Activated HIF-1α translocate to the nucleus and directly upregulates LAMA3 and ITGB1 mRNA expression. In turn, activated HIF-1α enhances Akt expression via positive feedback loop, as described previously [28]. Akt and mTOR induces IKK phosphorylation, followed by IKK degradation. Consequently, activated NF-κB/cyclin D pathway accelerates keratinocyte proliferation.

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Conflicts of Interest

The author declares that he has no conflicts of interest.

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