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# Effects of sulfated hyaluronan on keratinocyte differentiation and Wnt and Notch gene expression

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**Abstract**

Sulfated hyaluronan (SHya), which is composed of a sulfated group and hyaluronan (Hya), has high activity on and biocompatibility with cells. When normal human epidermal keratinocytes (NHEKs) were incubated in dishes coated with SHya, cell proliferation was suppressed in a dose-dependent manner. The expression levels of keratin 1 and loricrin mRNAs, as detected by real-time RT-PCR, were increased significantly. The expressions of Wnt mRNAs, which play important roles in cell proliferation and differentiation, were modulated. Wnt4 and Wnt6 mRNA expressions were increased compared to controls, while expression of Wnt5a was similar to the control and that of Wnt7a mRNA was decreased. In addition, the expression of Notch mRNAs, which play a critical role in keratinocyte differentiation, were affected. Notch3 mRNA was increased significantly, while Notch1 mRNA was decreased compared to controls, and expression of Notch2 was similar to that of control. These results suggested that a SHya-coated scaffold might be useful for regulating cell activity in tissue engineering.


*Keywords:* Sulfated hyaluronan; Normal human epidermal keratinocyte; Differentiation; Wnt; Notch

## 1. Introduction

Normal human epidermal keratinocytes (NHEKs) are usually cultured on 3T3 mouse feeder-layer cells [1]. Several biomaterials have been developed from other animals or humans, but they carry the risk of infection from prions and viruses [2,3]. In contrast, biomaterials from microorganisms carry lower risks of infection, and the development of a semi-synthetic material promoting cell activity will enable safer cell culture.

Several types of polysaccharides for culturing NHEKs have been studied [4,5]. Hyaluronan (Hya) is a negatively charged glycosaminoglycan that is a major component of the extracellular matrix (ECM) [6,7]. Hya plays important roles in cell adhesion, migration, proliferation, and differentiation [7–9]. Park and Tsuchiya reported that a Hya-coated surface is capable of enhancing gap junctional intercellular communication (GJIC) and differentiation or cell growth [10,11]. Sulfated polysaccharides, such as heparin or heparin sulfate, stabilize some growth factors, resulting in enhancement of their effects [12], and promote their mitogenic activity [13]. Sulfated hyaluronan (SHya), a semi-synthetic material composed of Hya and a sulfate group [14] can be synthesized using Hya extracted from microorganisms; therefore, it has a lower infectivity and a lower risk of containing virus-induced carcinogens.

It has been reported that Hya and chondroitin sulfate A enhanced chondrogenesis of human mesenchymal stem cells [15,16]. We hypothesized that SHya has the potential to function as a biomaterial promoting keratinocyte differentiation because it has been reported that SHya stimulates cell activities [14,17]. Analysis of the effects of SHya on cell differentiation and intercellular signaling will provide the information allowing construction of biomaterials of greater usefulness for tissue engineering.

Wnts are secreted glycoproteins that bind Frizzled receptors and play a critical role in the process of cell differentiation in the canonical pathway. In the canonical

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pathway, beta-catenin stabilized by the binding of Wnt proteins to Frizzled receptors stimulates TCF/LEF transcription. A mouse keratinocyte cell line with decreased Wnt4 expression showed a more malignant morphology and was less differentiated [18], Wnt6 was required for epithelialization of the segmental plate mesoderm [19], and Wnt7a promoted cell proliferation by activation of Rac-GTPase and beta-catenin [20].

Notch receptors play a crucial role in determination of cell fate. Notch1 signaling plays an essential role in regulation of mouse keratinocyte differentiation [21]. In mouse keratinocytes, Notch1 activation suppressed Wnt4 activity mediated by the cyclin/CDK inhibitor p21 (WAF1/Cip1) [22]. Notch3 was required for the differentiation of vascular smooth muscle cells and T-cells [23,24].

In a previous study, we demonstrated that a Hya coating promoted several cell functions better than a Hya-supplemented one [25]. Therefore, in this study, we investigated the effects of a SHya coating on keratinocyte differentiation.

## 2. Materials and methods

### 2.1. Sulfated hyaluronan

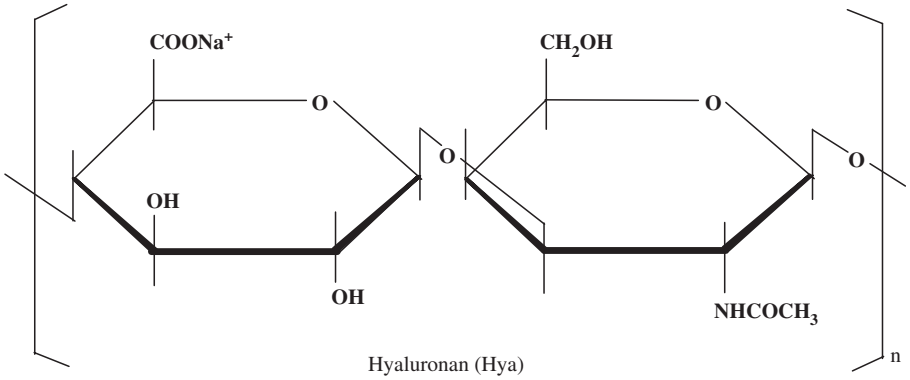
SHya was prepared by the method reported previously [4]. A solution of 2% Hya120 (molecular weight, $1.2 \times 10^6$) solution in *N*,*N*-dimethylformamide (DMF) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was mixed with trimethylamine (TMA)–$SO_3$ complex (Aldrich Chemical Co., Inc., Milwaukee, WI, USA) and stirred for 24 h at 60 °C. The reaction mixture was then diluted, neutralized, and precipitated by adding a large quantity of acetone (Wako Pure Chemical Industries). The precipitate was dissolved in distilled water and dialyzed against distilled water. The molecular weight of SHya was $2.0 \times 10^5$, and the degree of substitution (D.S.) of SHya was 1.0, as determined by the chelate titration method [26] (Fig. 1). Moreover, the effectiveness of sulfation was also demonstrated by FT-IR analysis. The IR spectrum of SHya exhibited two absorption bands at 1240 and 820 $cm^{-1}$ due to S=O and $SO_3^-$ stretching, respectively.

### 2.2. Cell culture

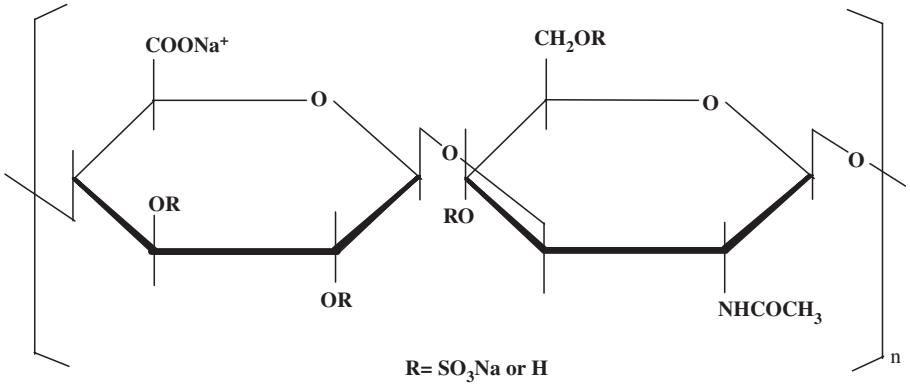
NHEKs isolated from neonatal human foreskins (Cambrex Bioscience, Walkersville, MD, USA) were cultured with K-110 Type II medium (Kyokuto, Tokyo, Japan) supplemented with 2% whole bovine pituitary extract, 50 IU penicillin G, 50 μg/ml streptomycin, and 0.03 mM $CaCl_2$ (low-calcium condition) at 37 °C in a humidified atmosphere of 5% $CO_2$ and 95% air. Keratinocyte differentiation was induced in 0.20 mM $CaCl_2$ medium (high-calcium condition). The medium was exchanged for a fresh one every 2 days.

### 2.3. Preparation of SHya-coated culture dishes and well plates

Both 35 and 100 mm polystyrene dishes (Iwaki, Funabashi, Japan) and 24-well plates (Corning, Corning, NY, USA) were coated with SHya dissolved in distilled water at a final density of 0.4 or 0.8 mg/$cm^2$. The SHya-coated dishes and plates were dried under a sterile airflow at room temperature for 8 h.




Hyaluronan (Hya)




Sulphated hyaluronan (SHya)

Fig. 1. The structures of hyaluronan and sulfated hyaluronan. Sulfated hyaluronan (SHya) is composed of Hya and a sulfate group. The molecular weight of SHya is $2.0 \times 10^5$, and the degree of substitution of SHya was 1.0.

### 2.4. Determination of cell numbers by crystal violet assay

NHEKs were seeded at $1.0 \times 10^4$ cells/cm$^2$ in wells of 24-well plates coated with various concentrations of SHya (0, 0.4, and 0.8 mg/cm$^2$) and incubated in the high-calcium condition (0.20 mM) for 5 days. After washing with $Ca^{2+}$, $Mg^{2+}$-free phosphate-buffered saline [PBS(−)], cells were exposed to 0.4% crystal violet (Wako) in methanol for 15 min. NHEKs were washed with PBS(−) three times and destained with 500 μl of methanol for 20 min. Then, absorbance was read at 590 nm using a plate reader.

### 2.5. Quantitative real time RT-PCR

NHEKs were seeded at $1.0 \times 10^4$ cells/cm$^2$ in various concentrations on SHya-coated 60 mm dishes (0, 0.4, and 0.8 mg/cm$^2$) and incubated in the high-calcium condition (0.20 mM) for 5 days. Cells were washed with PBS(−) three times, and total RNA was extracted from NHEKs using an RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA was reverse transcribed into 10 μl DNA using an ExScript RT reagent kit (Takara Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. Aliquots of the cDNA were used as templates for PCR analysis using a Lightcycler system (Roche, Mannheim, Germany). PCR amplification was performed in a total volume of 20 μl including 1 μl of RT reaction, 10 μl of SYBR *Premix Ex Taq* (Takara), and 0.4 μM/l of each primer. The PCR reaction was performed as follows: 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 65 °C for 15 s. The PCR primers were purchased from Takara Co., Ltd. The PCR primer sequences for amplification of keratin 1 were forward primer 5′-AGATCACTGCTGGCAGACATGG-3′, and reverse primer 5′-TGATGGACTGCTGCAAGTTGG-3′. The PCR primer sequences for amplification of loricrin were forward primer 5′-TCATGATGC-TACCCGAGGTTTG-3′, and reverse primer 5′-CAGAACTAGATG CAGCCGGAGA-3′. The PCR primer sequences for amplification of Wnt4 were forward primer 5′-CCAGCAGAGCCCTCATGAAC-3′, and reverse primer 5′-TCCACCTCAGTGGCACCATC-3′. The PCR primer sequences for amplification of Wnt6 were forward primer 5′-CTG GAATTGCTCCAGCCACA-3′, and reverse primer 5′-GCAGTGAT GGCGAACACGA-3′. The PCR primer sequences for amplification of Wnt7a were forward primer 5′-GCCCGGACTCTCATGAACTTG-3′, and reverse primer 5′-CCTCGTTGTACTTGTCCTTGAGCA-3′. The PCR primer sequences for amplification of Notch1 were forward primer 5′-TGCGAGGTCAACACAGACGAG-3′, and reverse primer 5′-GTG TAAGTGTTGGGTCCGTCCAG-3′. The PCR primer sequences for amplification of Notch2 were forward primer 5′-TGAACACTGGGTC GATGATGAAG-3′, and reverse primer 5′-AGCGATGGTGTCC TACGGATG-3′. The PCR primer sequences for amplification of Notch3 were forward primer 5′-TGATGGCATGGATGTCAATGTG-3′, and reverse primer 5′-CAGTTGGCATTGGCTCCAGA-3′. The PCR primer sequences for amplification of GAPDH were forward primer 5′-GCACCGTCAAGGCTGAGAAC-3′, and reverse primer 5′-ATGG TGGTGAAGACGCCAGT-3′. Each sample was tested in triplicate.

### 2.6. Statistical analysis

Significant differences between groups were evaluated with Student's *t*-test. Mean differences were considered significant when $^{**}p < 0.01$. Three samples were run for each case. All experiments were repeated at least twice, and similar results were obtained.

## 3. Results

### 3.1. Adhesiveness of NHEKs to SHya

Normal human dermal fibroblasts showed low adhesion to Hya-coated surfaces [9,10]. In order to assess the adhesion of NHEKs to SHya-coated surfaces, NHEKs were seeded on SHya- and Hya-coated surfaces (0, 0.4, and 0.8 mg/cm$^2$). Fig. 2 shows that NHEKs adhered to SHya-coated surfaces as well as to an uncoated dish, but they did not adhere to the Hya-coated dishes.

### 3.2. Effect of SHya coating on keratinocyte proliferation

NHEKs were seeded on SHya-coated dishes (0.4 or 0.8 mg/cm$^2$) and incubated in a high-calcium condition

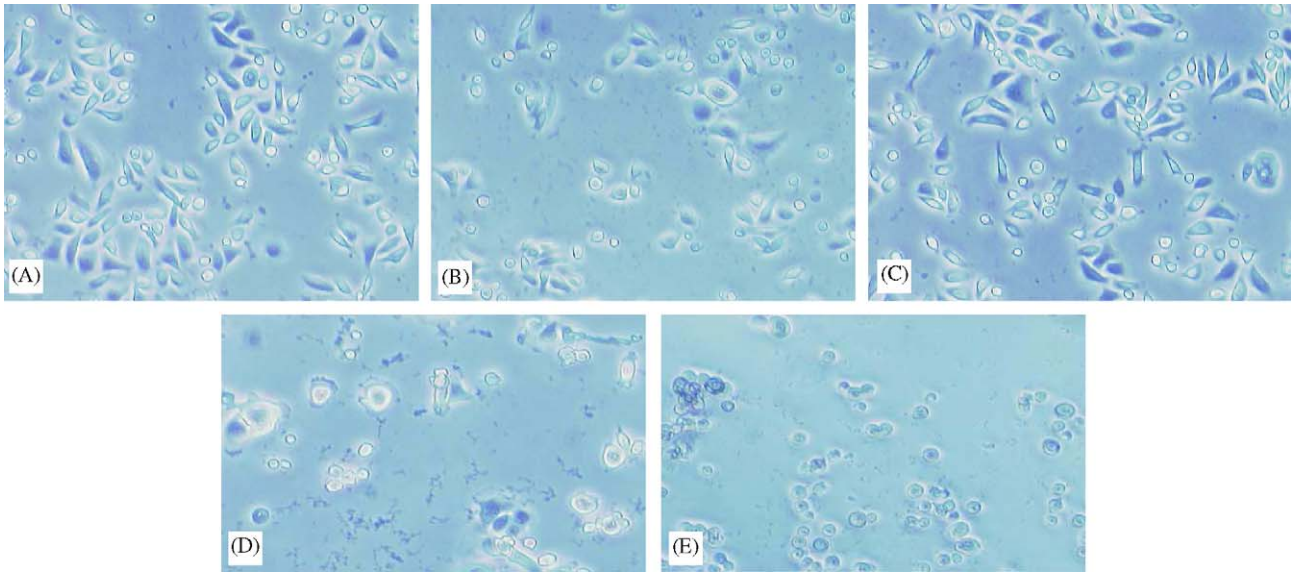



Fig. 2. The adhesion of NHEKs to SHya-coated surfaces. NHEKs were seeded at $1.0 \times 10^4$ cells/cm$^2$ onto SHya- and Hya-coated 24-well plates. Then NHEKs were incubated with non-coated (A), 0.4 mg/cm$^2$ SHya-coated (B), 0.8 mg/cm$^2$ SHya-coated (C), 0.4 mg/cm$^2$ Hya-coated (D), and 0.8 mg/cm$^2$ Hya-coated (E) wells for 16 h.

(0.20 mM) for 5 days. The cell proliferation assay showed that the SHya coating suppressed keratinocyte proliferation remarkably in a dose-dependent manner (Fig. 3).

### 3.3. Effect of SHya coating on keratin1 and loricrin expression

To ensure that the SHya coating promoted keratinocyte differentiation, we detected the relative expression levels of differential marker mRNA by real-time RT-PCR. After NHEKs were incubated with SHya coating in a high-calcium condition (0.20 mM) for 5 days, the expression level of keratin1 mRNA on NHEKs was increased more than eight-fold compared to the control (Fig. 4A) and that of loricrin mRNA was increased in a dose-dependent manner (Fig. 4B).

### 3.4. Effect of SHya coating on Wnts expressions

The expression levels of Wnt4 and Wnt6 mRNA on NHEKs incubated with SHya coating in the low-calcium condition were measured (Fig. 5A and B); the expression level of Wnt5a of NHEKs incubated with SHya coating was decreased to 95% of the control (Fig. 5C) and that of Wnt7a mRNA to about 40% of the control (Fig. 5D).

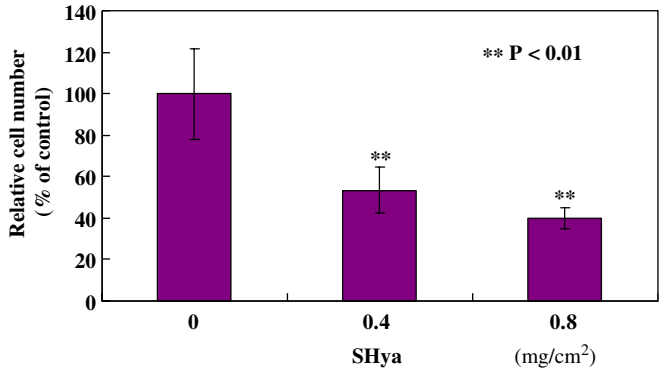



Fig. 3. Suppressive effect of SHya on keratinocyte proliferation. NHEKs were seeded at $1.0 \times 10^4$ cells/cm$^2$ densities onto non-coated (A), 0.4 mg/cm$^2$ SHya-coated (B), and 0.8 mg/cm$^2$ SHya-coated (C) 24-well plates and cultured for 5 days. Then numbers of NHEKs were determined by crystal violet assay. Each value is expressed as the mean ± SD. $^{**}P < 0.01$ compared to control.

### 3.5. Effect of SHya coating on Notch expressions

The expression level of Notch1 mRNA on NHEKs incubated with SHya coating was decreased about 75% compared to the control, and that of Notch2 mRNA was similar to the control (Fig. 6A and B). However, the expression level of Notch3 mRNA was increased about eight-fold compared to the control (Fig. 6C).

## 4. Discussion

Several studies have suggested that SHya interacts with cells [4], but the effect of SHya on cell differentiation and intercellular signaling was not clear. We demonstrated that a SHya coating promoted keratinocyte differentiation and modulated the expression levels of Notch and Wnt mRNAs.

In this study, the expression levels of Wnt4 and Wnt6 on NHEKs incubated with SHya were increased. A mouse keratinocyte cell line with a deficit of Wnt4 expression showed less differentiation [18]. Wnt6 regulated epithelization [19], suggesting that the SHya-induced upregulation of Wnt4 and Wnt6 is associated with the regulation of keratinocyte differentiation. Wnt7a promoted cell proliferation in corneal epithelial cells during wound healing [20], suggesting that SHya down-regulated Wnt7a expression, resulting in the enhancement of keratinocyte differentiation. The activation of beta-catenin, a downstream factor of Wnt signaling, contributes to keratinocyte differentiation [27]. A sulfated proteoglycan-induced Wnt-11 expression in mouse kidney cells, and sulfated polysaccharides were required in Wnt signaling in mouse kidney cells [28,29]. Therefore, it was suggested

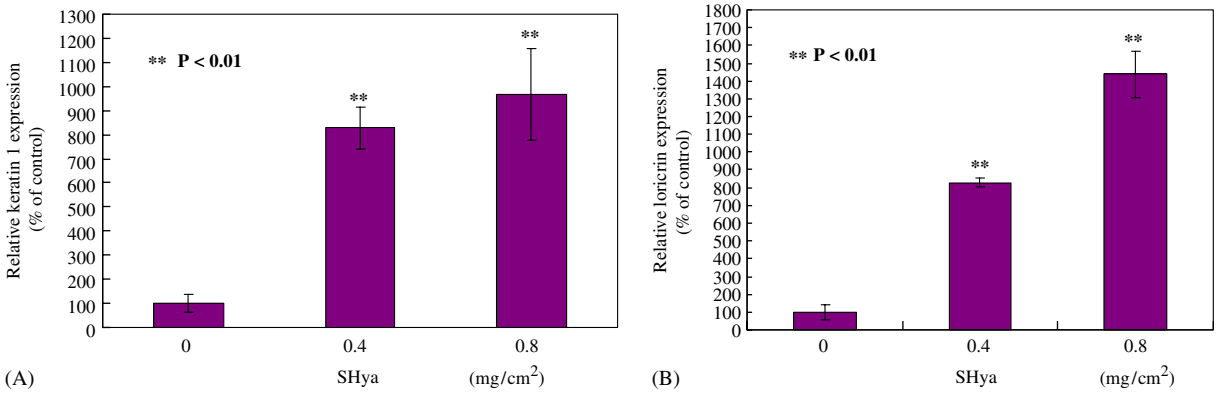



Fig. 4. The expression levels of keratin1 and loricrin mRNA of NHEKs incubated with SHya coating. NHEKs were seeded at $1.0 \times 10^4$ cells/cm$^2$ onto SHya-coated 60 mm dishes (0, 0.4, and 0.8 mg/cm$^2$) and cultured in medium with 0.20 mM calcium for 5 days. Then RNA was extracted, and real-time RT-PCR was performed to determine the expression levels of keratin1 and loricrin mRNA. Effect of SHya on the expression level of (A) keratin1 mRNA, and (B) loricrin mRNA. Each value is expressed as the mean ± SD. $^{**}P < 0.01$ compared to control.

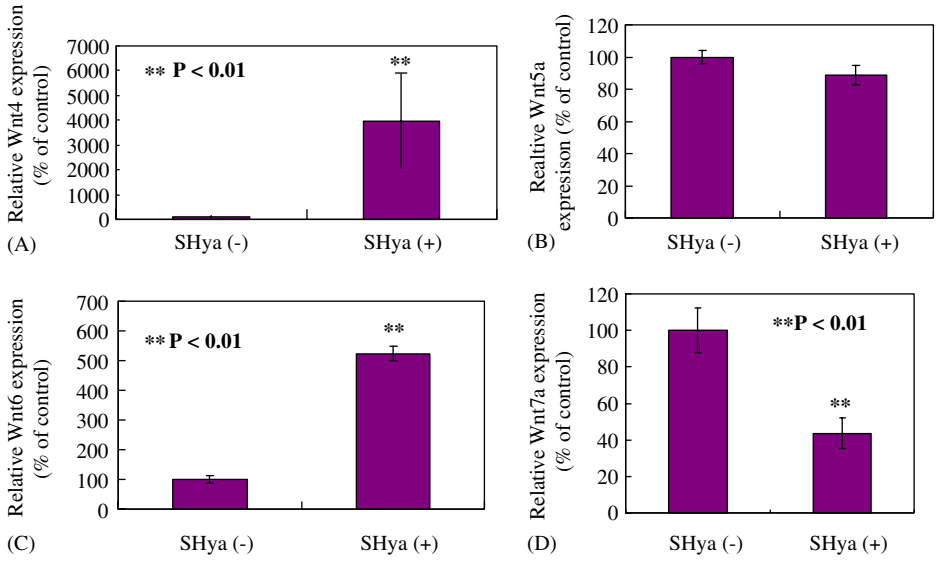



Fig. 5. The expression level of Wnt mRNA in NHEKs incubated with SHya coating. NHEKs were seeded at $1.0 \times 10^4$ cells/cm$^2$ onto SHya-coated 60 mm dishes (0 and 0.8 mg/cm$^2$) and cultured in medium with 0.20 mM calcium for 20 h. Then RNA was extracted, and real-time RT-PCR was performed to determine the expression level of Wnt mRNA. Effect of SHya on the expression level of (A) Wnt4 mRNA, (B) Wnt5a mRNA, (C) Wnt6 mRNA, and (D) Wnt7a mRNA. Each value is expressed as the mean ± SD. $^{**}P < 0.01$ compared to control.

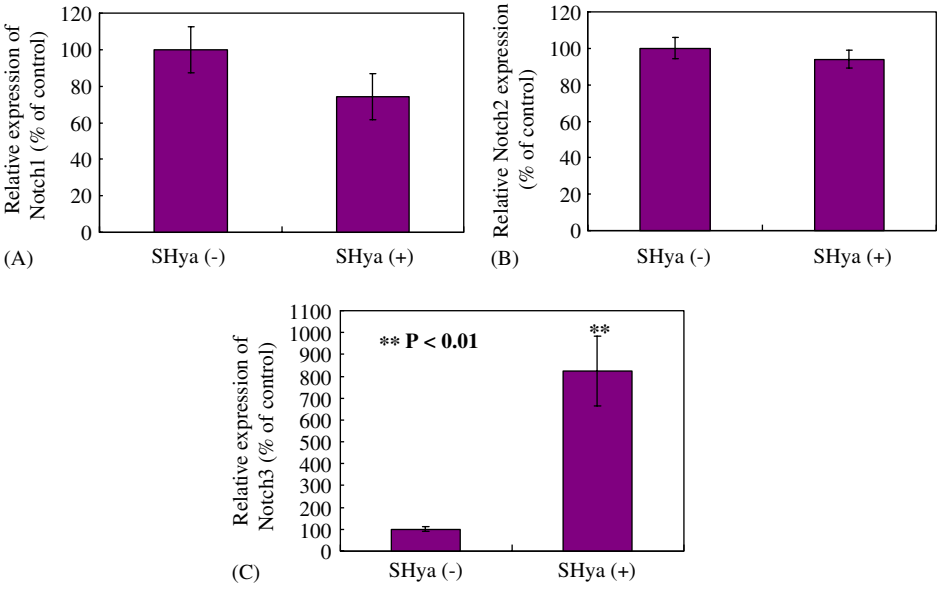



Fig. 6. The expression level of Notch mRNA in NHEKs incubated with SHya coating. NHEKs were seeded at $1.0 \times 10^4$ cells/cm$^2$ onto SHya-coated 60 mm dishes (0, 0.4, and 0.8 mg/cm$^2$) and cultured in medium with 0.20 mM calcium for 5 days. Then RNA was extracted, and real-time RT-PCR was performed to measure the expression level of notch mRNA. The effect of SHya on the expression level of (A) Notch1 mRNA, (B) Notch2 mRNA, and (C) Notch3 mRNA. Each value is expressed as the mean ± SD. $^{**}P < 0.01$ compared to control.

that SHya modulated Wnt signaling leading to beta-catenin activation.

In mouse keratinocytes, Notch1 is associated with the regulation of cell differentiation via p21, and Notch1 activation down-regulates Wnt4 expression [21]. Notch1 is required in keratinocyte differentiation and in the regulation of Wnt expression [24]. Activation of Notch receptors induces an increase in its own expression level by a positive feedback mechanism [24]. The expression of Notch1 in NHEKs incubated in SHya-coated dishes was decreased compared to that of the control. It was suggested that the decrease in Notch1 expression triggered by SHya induced the increase of Wnt4. The expression level of Notch3 mRNA in NHEKs incubated with SHya coating was increased, suggesting that SHya interacted with Notch3, particularly resulting in the modulation of Wnt expression (Fig. 7). Notch3 is required for the differentiation of vascular smooth muscle cells or T cells [23,24]. The role of Notch3 in keratinocyte differentiation triggered by elevation of the extracellular calcium-ion concentration may not

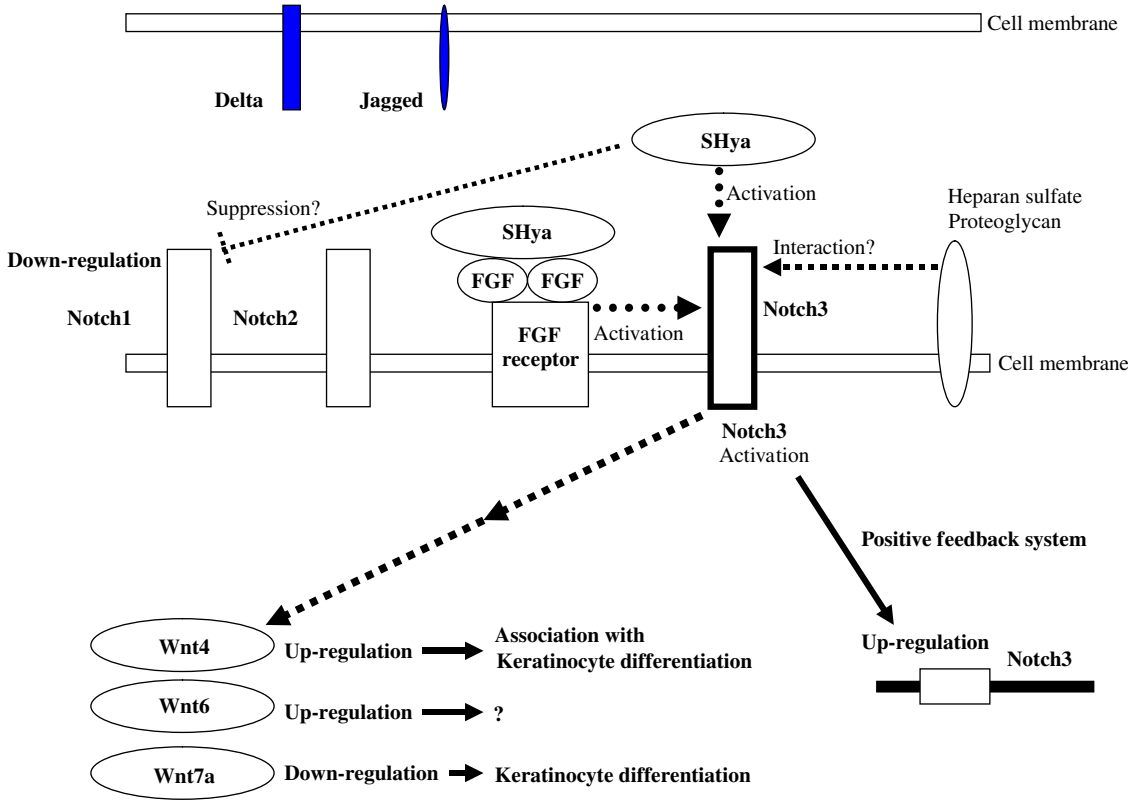



Fig. 7. Diagram of the effect of SHya on Wnt expression via Notch3.

be important, but Notch3 may be activated by SHya, leading to a modulation of intracellular signaling and enhancement of keratinocyte differentiation. Notch3 may be required for interaction with the sulfate groups of sulfated polysaccharides.

Notch1 activation stimulates p21 via the RBP-J kappa transcription factor, resulting in growth arrest, keratinocyte differentiation, or a decrease of Wnt4 expression [25]. Therefore, SHya might activate p21 via Notch3 activation, leading to the modulation of Wnt expression.

The differentiation of NHEKs incubated in SHya was better than that in Hya (data not shown). This suggests that the introduction of sulfate groups into Hya may be a key factor in the enhancement of keratinocyte differentiation.

Normal human dermal fibroblasts showed very low adhesiveness to Hya-coated surfaces because of the anionic surface of Hya [10,11]. However, NHEKs showed very high adhesiveness to SHya-coated surfaces. This suggested that the introduction of sulfate groups into Hya may change the property of its surface, resulting in a high adhesiveness. Sulfated polysaccharides such as heparin and heparan sulfate enhance the stabilities of some growth factors or adsorption of them by the cell membrane, resulting in the enhancement of cell differentiation [13]. The sulfate groups of SHya may bind to and stabilize cationic growth factors, and stabilized growth factors may neutralize the negative charge of the SHya surface, resulting in high cell attachment. Further, it was reported that fibroblast growth factors (FGF) receptors interact with the Notch signaling pathway [30,31]. Therefore, it was suggested that SHya binds and stabilizes FGF to activate FGF receptors leading to stimulation of Notch3 and intercellular signaling.

## 5. Conclusion

This study demonstrated that a SHya coating promoted keratinocyte differentiation triggered by an elevated extracellular calcium ion concentration. Furthermore, SHya modulated Wnt expressions and increased the expression level of Notch3 mRNA. These results suggest that Notch3 may be an important target for the regulation of cell differentiation. SHya may be a useful biomaterial to regulate Wnt signaling in tissue engineering. This study provides new information that clarifies the interaction between sulfate groups and Notch families. Studies are in progress to clarify the roles of these modulations of *Notch* and *Wnt* genes in keratinocyte differentiation.

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