B-Myb Enhances Proliferation and Suppresses Differentiation of Keratinocytes in Three-dimensional Cell Culture

（B-Myb は表皮角化細胞の三次元培養において増殖を促進し、分化を抑制する）

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Abstract

B-Myb (Mybl2) is a member of the *Myb* gene family of transcription factors involved in the control of cell growth, differentiation, and apoptosis. The effects of B-Myb on keratinocyte proliferation and differentiation have not yet been clarified. The present study was performed to examine the role of B-Myb in proliferation and differentiation of the spontaneously immortalized human skin keratinocyte cell line HaCaT and normal human keratinocytes (NHK) with formation of a stratified epidermoid structure in air–liquid interface 3-dimensional culture. B-Myb was expressed specifically in undifferentiated normal keratinocytes and downregulated during differentiation. The constitutive overexpression of B-Myb in HaCaT cells during air exposure-induced differentiation resulted in an undifferentiated phenotype, i.e., thickening of the stratified layers, suppression of differentiation marker expression, and retention of proliferative activity with activation of cell cycle regulatory proteins in the S and G2/M phases. In contrast, suppression of B-Myb caused their downregulation and constrained proliferation with retention of differentiation capacity. These findings suggested that B-Myb may play an important role in maintenance of the undifferentiated phenotype of keratinocytes in the basal epidermal layer.

Introduction

The epidermis is a self-renewing tissue that provides a useful system in which to study the processes involved in cell proliferation and differentiation. An elaborate and defined program of differentiation is involved in the structural and functional maturation of epidermal keratinocytes. Undifferentiated keratinocytes in the basal layer have high rates of proliferative activity, and these cells undergo cell cycle arrest and achieve differentiation as they move up to the suprabasal layers. Analyses of epidermal differentiation identified a set of genes that are transcribed only during certain stages of differentiation. Keratinocytes in the basal layer express keratin 5 (K5) and keratin 14 (K14) (1). Upon maturation in the spinous layer, K5 and K14 are downregulated and the genes encoding keratin 1 (K1) and keratin 10 (K10) are induced (2) (3). As these cells differentiate into granular layer cells, they cease expression of K1 and K10 and show induction of late-differentiation products, such as filaggrin (4) (5), involucrin (6), and loricrin (7).

B-Myb (Mybl2) is a member of the *Myb* gene family of transcription factors involved in the control of cell growth, differentiation, and apoptosis. The *Myb* gene family consists of three members: c-Myb, A-Myb, and B-Myb (8). B-Myb is ubiquitously expressed,

whereas c-Myb and A-Myb have restricted tissue-specific expression patterns (9). A-Myb is expressed primarily in B-cells, developing nerve cells, and reproductive tissues, while c-Myb is mainly expressed in hematopoietic precursors (10). The level of B-Myb protein is proportional to the degree of cell proliferation (11). B-Myb has been shown to be overexpressed in prostate cancer (12) and basal-like breast cancer (13), and it is involved in the poor prognosis of these diseases. Cyclin D1 quenches B-Myb transactivation by interfering with CBP/p300 until cyclin D1 destruction in late G1 phase. From late G1 phase, B-Myb is phosphorylated and activated by cdk2 and cyclin A/E. B-Myb stimulates the transcription of genes that promote entry into the G2/M phases of the cell cycle (14) (11) (15). In terms of proliferation and differentiation, B-Myb is downregulated during colon epithelial cell maturation (16). However, the role of B-Myb in cell cycle progression is dependent on cell type (17).

In the present study, I examined the role of B-Myb in differentiation of the spontaneously immortalized human skin keratinocyte cell line HaCaT and normal human keratinocytes (NHK) with formation of a stratified epidermoid structure in air–liquid interface 3-dimensional (3D) culture.

Material & Methods

Plasmids

The plasmid pAct-B-Myb carrying human B-Myb DNA was a kind gift from Dr. Shunsuke Ishii (Laboratory of Molecular Genetics, RIKEN Tsukuba Institute, Tsukuba, Japan). The pCMV expression vector pCMV-B-Myb was derived from pRC/CMV empty vector (Invitrogen Life Technologies, Carlsbad, USA) by subcloning the *Sac*II/*Xba*I fragment from pAct-B-Myb. The construct was confirmed by enzymatic digestion and sequencing before use.

Cell culture

HaCaT cells were maintained in MCDB153 medium (Sigma Aldrich, St. Louis, USA) supplemented with 0.1 mM calcium chloride, 10 ng ml^{-1} epidermal growth factor (EGF) (Sigma Aldrich), 1% penicillin-streptomycin solution (Sigma Aldrich), and 5% dialyzed fetal bovine serum (Gibco-BRL, Paisley, UK) as described previously (18). Primary mouse keratinocyte cultures were prepared as described previously (19). Normal human keratinocytes (NHKs) (FC-0007, Lifeline cell technology, Frederick, USA) were

cultured in Dermalife K medium complete kit (Lifeline cell technology) in accordance with the manufacturer's instructions. 3D cell culture was performed as described previously (20).

DNA transfection

HaCaT cells were transfected using the TransIT-LT1 Transfection Reagent (Mirus Bio, Madison, USA) in accordance with the manufacturer's recommendations. DNA complex was prepared using 1 μg of plasmid, 3 μl of LT1, and Eagle's minimal essential medium. Stable HaCaT clones transfected with pCMV-B-Myb and with empty pCMV vector alone were selected and maintained in the presence of 300 μg ml^{-1} of the aminoglycoside antibiotic G418 (Geneticin; Gibco-BRL). Single colonies were cloned from one HaCaT cell, and the cells were proliferated in 24-well plates or in dishes 10 cm in diameter.

Immunoblotting

Cells were solubilized using Sample Buffer Solution (Wako, Osaka, Japan). The whole-cell lysates were subjected to SDS-PAGE on Mini-PROTEAN TGX Precast Gels (Bio-Rad, Hercules, USA), and the proteins were transferred onto nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK). The membranes were incubated overnight with primary antibodies against B-Myb (1 mg ml^{-1}; Ab-577/581; Assaybiotech, San Francisco, USA), keratin 10 (0.2 mg ml^{-1}; PRB-159P; Covance, Emeryville, USA), cyclin A (10 mg ml^{-1} RB-1548; Thermo Fisher Scientific, Newark, DE), cyclin B1 (05-373SP; Millipore, Billerica, USA), β-actin (0.2 mg ml^{-1}; BioVision, Mountain View, USA) and CDK1 (CDC2; 0.2 mg ml^{-1}; sc-54; Santa Cruz Biotechnology), followed by incubation for 60 min with horseradish peroxidase–labeled secondary antibodies against rabbit IgG (0.04 mg ml^{-1}; Santa Cruz Biotechnology) and mouse IgG (0.4 mg ml^{-1}; Santa Cruz Biotechnology). Antibody binding was visualized and enhanced with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and Hyperfilm MP (GE Healthcare).

Measurement of thickness of epidermal layers

Sections of 3D cultured cells stained with hematoxylin and eosin were examined. The thickness of the epidermal layer was measured using a digital microscope (AX80; Olympus, Tokyo, Japan). Ten points were selected at random and measured from the basal layer to the horny layer on vertical sections.

Immunohistochemical staining

Sections of 3D cultured cells were pretreated by autoclaving in 10 mM citrate buffer (pH 6.0) before immunostaining for antigen retrieval. Cells were immunostained for p-B-Myb (ab76009; Abcam, Cambridge, UK), loricrin (PRB-145P; Covance), involucrin (MS-126; Thermo Fisher Scientific), keratin 10 (ab76318; Abcam), and Ki-67 (ab833; Abcam). All of the following procedures were carried out in accordance with standard protocols using an En Vision kit (Dako) and sections were lightly counterstained with hematoxylin.

Quantitative real-time RT-PCR Analysis

Total RNA was extracted from culture dishes 10 cm in diameter at 80% – 90% confluency using ISOGEN (Nippon Gene, Tokyo, Japan), and complementary DNA (cDNA) was synthesized using a Prime Script RT Reagent kit (Takara Bio, Shiga, Japan). An ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, USA) was used for SYBR Green (Takara Bio)-based quantitative analysis. The quantity of mRNA was normalized relative to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Quantification was performed in triplicate. The primers used were as follows:

B-Myb (human) (forward primer: 5'-GTAACAGCCTCACGCCCAAGA; reverse primer: 5'-GTGTCCTGTTTGTTCCAGAAGTT), B-Myb (mouse) (forward primer: 5'-GTGTGGCCCATAGCTGGTG; reverse primer: 5'-CACCCAGGCAAGGCTACTG), keratin 10 (mouse) (forward primer: 5'-CTGACAATGCCAACGTGCTG; reverse primer: 5'-GCAGGGTCACCTCATTCTCGTA), Cyclin A1 (human) (forward primer: 5'-GAAATTGTGCCTTGCCTGAGTG; reverse primer: 5'-TCTGATATGGAGGTGAAGTTCTG), CDK1 (human) (forward primer: 5'-GTGGCCAGAAGTGGAATCTTTAC; reverse primer:

5'-CGTTTGGCTGGATCATAGATTAACA), CDK2 (human) (forward primer: 5'-CATGGATGCCTCTGCTCTCAC; reverse primer: 5'-GACCCGATGAGAATGGCAGAA), GAPDH (human) (forward primer: 5'-GCACCGTCAAGGCTGAGAAC; reverse primer: 5'-TGGTGAAGACGCCAGTGGA), GAPDH (mouse) (forward primer: 5'-AAATGGTGAAGGTCGGTGTGAAC; reverse primer: 5'-CAACAATCTCCACTTTGCCACTG).

Silencing of B-Myb expression in HaCaT cells

Lentiviral particles encoding shRNA targeting B-Myb mRNA were purchased from Santa Cruz Biotechnology (sc-43523-V). Lentiviral particles encoding a scrambled shRNA sequence (sc-108080) were also purchased from the same source for use as a control. HaCaT cells and NHKs were transduced with these lentiviral particles according to the manufacturer's instructions, and the knockdown efficacy was assessed at the protein level by Western blotting.

Measurement of NHKs proliferation rate

NHKs of each strain were seeded into six-well plates at a density of 5×10^4 cells per well in the growth medium. The next day, the medium was replaced with fresh medium. The numbers of cells in triplicate wells were counted by trypsinization after 48 and 96 hours after seeding.

Statistical analysis

Data are expressed as means ± SD. Comparisons among groups were carried out using the paired Student's *t* test. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

Results

B-Myb expression is downregulated during normal keratinocyte differentiation

Normal neonatal murine keratinocytes were cultured in medium containing a low or high calcium concentration. Normal murine keratinocytes (NMKs) cultured under high-calcium conditions undergo a phenomenon known as the calcium switch, which involves cell cycle exit followed by terminal differentiation (19). The results of quantitative real-time PCR indicated significantly elevated B-Myb expression in undifferentiated keratinocytes cultured under low-calcium conditions (0.05 mM calcium) compared to differentiated keratinocytes cultured under high-calcium conditions (0.12 mM calcium) at the mRNA level (Figure 1a). In addition, the results of immunoblotting analyses indicated significantly elevation of B-Myb expression in undifferentiated keratinocytes compared to differentiated keratinocytes at the protein level (Figure 1b). Therefore, these findings indicated that B-Myb is expressed specifically in undifferentiated keratinocytes and downregulated during their differentiation.

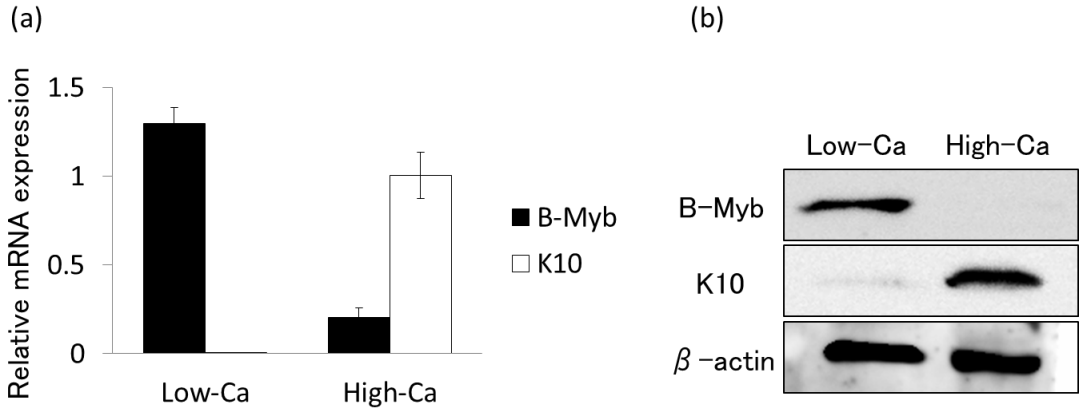

Figure 1

Expression of B-Myb in the normal keratinocytes.

(a) Total RNAs extracted from neonatal murine keratinocytes (NMKs) cultured in medium containing low or high levels of calcium were subjected to quantitative real-time PCR. Relative expression of B-Myb and keratin 10 (K10) mRNAs in NMKs under low- and high-calcium conditions. B-Myb mRNA expression level was 6.3-fold higher under low-calcium conditions than under high-calcium conditions. The results indicated cell cycle-dependent transcriptional regulation of B-Myb. The expression levels were normalized relative to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. All values represent means ± SD from three independent experiments. $^{**}P < 0.01$. (b) Immunoblotting of whole-cell extracts from NMKs

cultured under low- or high-calcium conditions. B-Myb protein expression became almost undetectable as NMKs underwent differentiation.

HaCaT cells overexpressing B-Myb formed an epidermoid epithelium with increased thickness in 3D culture

The 3D cultures of control HaCaT cells (Figure 2b, left column) and those overexpressing B-Myb (Figure 2b, right column) grown for 4, 7, 10, and 14 days showed layers mimicking the epidermal structure. The mean thicknesses of the epithelial layers were measured after 4, 7, 10, and 14 days of culture (Figure 2c). The cultures of HaCaT-B-Myb cells became thicker than those of cultures of HaCaT-control cells on days 4, 7, 10, and 14. These observations indicated that B-Myb accelerates the cell cycle and cell proliferation. This was supported by the results of immunostaining for Ki-67 (Figure 2d). On days 4 and 7, cultures of HaCaT-B-Myb and HaCaT-control cells showed appropriately equivalent numbers of Ki-67-positive cells. On the other hand, more Ki-67-positive cells were observed in cultures of HaCaT-B-Myb cells than HaCaT-control cells on days 10 and 14. These findings suggested that the overexpression of B-Myb was responsible for cell proliferation, thus resulting in an increase in thickness of the epithelia.

Overexpression of B-Myb in HaCaT cells suppressed epithelial keratinization and maintained the cells in an undifferentiated condition under 3D conditions

The effects of B-Myb on keratinocyte differentiation in 3D culture were analyzed by histological immunostaining of 3D cultures for several epithelial differentiation markers: i.e., loricrin as a marker of terminal differentiation, involucrin as a marker of late-stage differentiation, and K10 as a marker of early-stage differentiation. The levels of expression of the early-stage, late-stage and terminal differentiation markers K10, involucrin, and loricrin, respectively, were markedly decreased in B-Myb-overexpressing epithelia in comparison with the controls (Figure 2e). Positive immunoreactivity for the undifferentiated basal cell marker K14 was detected in all layers in both B-Myb-transfected and control epithelia. These findings indicated that overexpression of B-Myb in HaCaT cells functionally suppresses epithelial keratinization and maintains the cells in the undifferentiated state.

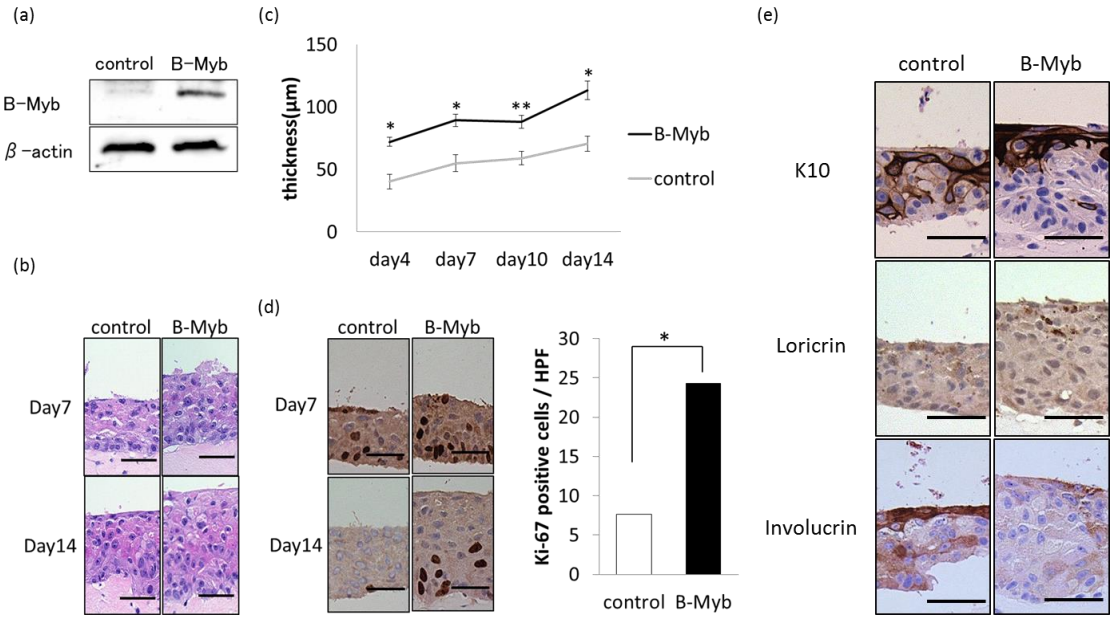

Figure 2

Effects of B-Myb overexpression on the morphology of three-dimensional (3D) cell cultures.

(a) Whole-cell lysates from HaCaT cells transfected with B-Myb expression vector or empty vector were analyzed with B-Myb protein expression by immunoblotting analysis. Control: control cultures. B-Myb: pCMV-B-Myb-treated cultures. (b) Hematoxylin and eosin (HE) staining of HaCaT 3D cell cultures. Control cultures, left column; pCMV-B-Myb-treated cultures, right column. Overexpression of B-Myb in

HaCaT cells increased the thickness of epithelial cell layers. (c) Thickness of epidermoid layers. All values represent means ± SD (n = 3). * P < 0.05 vs. control, ** P < 0.01 vs. control. (d) Immunostaining for Ki-67 in HaCaT 3D cell cultures. Control cultures, left column; pCMV-B-Myb-treated cultures, right column. Data are means ± SD (n = 3). *P < 0.05 vs. control. (e) Immunostaining for the differentiation markers K10, loricrin, and involucrin in HaCaT 3D cell cultures. Control cultures, left column; pCMV-B-Myb -treated cultures, right column. Bar = 50 μm.

ShRNA-mediated silencing of B-Myb in HaCaT cells inhibited epithelial thickening and decreased thickness in 3D culture

The expression of B-Myb was suppressed by lentiviral vector-expressed short hairpin RNA (shRNA) treatment. Control HaCaT cells (Figure 3b, left column) and sh-B-Myb HaCaT cells (Figure 3b, right column) were grown in 3D culture for 4 – 14 days. The controls were transduced with scramble lentiviral vector. The mean thicknesses of the epithelial layers were measured after 4, 7, 10, and 14 days of culture (Figure 3c). The cultures of sh-B-Myb HaCaT cells were not as thick as those of the control HaCaT cells on days 4, 7, 10, and 14. These observations were inversely related to the overexpression of B-Myb indicating that B-Myb is required to some extent for the cell cycle and cell proliferation. This was also supported by the results of immunostaining for Ki-67 (Figure 3d). That is, fewer Ki-67-positive cells were observed on days 4, 7, 10, and 14 in cultures of sh-B-Myb HaCaT cells than control HaCaT cells. These findings suggest that the suppression of B-Myb expression abrogated cell proliferation and resulted in a decrease in thickness of the epithelia. The suppression of B-Myb resulted in a decrease in thickness of undifferentiated layers, which do not express differentiation markers, such as K10 and involucrin (Figure 3e).

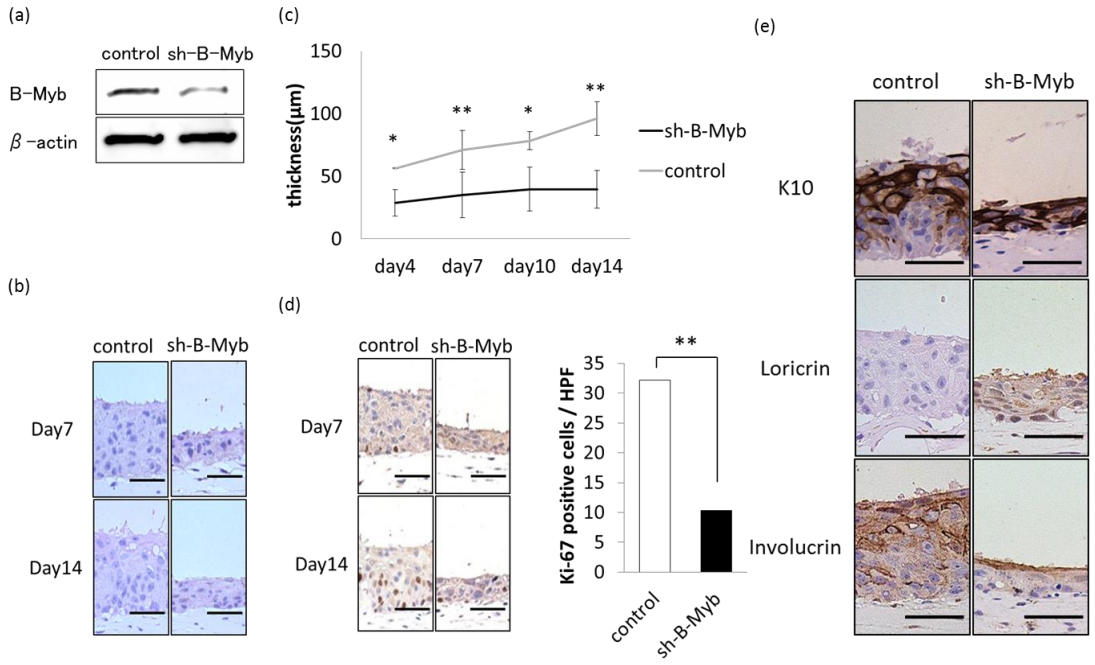

Figure 3

Effects of short hairpin RNA (shRNA)-mediated silencing of B-Myb expression on the morphology of three-dimensional (3D) cell cultures.

(a) Whole-cell lysates from HaCaT cells treated with lentiviral particles expressing shRNA targeting the B-Myb mRNA or non-target control were analyzed with B-Myb protein expression by immunoblotting analysis. Control: control cultures. sh-B-Myb: shRNA-treated cultures. (b) Hematoxylin and eosin (HE) staining of HaCaT 3D cell cultures. Control cultures, left column; shRNA-treated cultures, right column. Silencing

of B-Myb expression in HaCaT cells inhibited growing thick. (c) Thickness of epidermoid layers. All values represent means ± SD (n=3). * $p<0.05$ vs. control. ** $p<0.01$ vs. control. (d) Immunostaining for Ki-67 staining of HaCaT 3D cell cultures. Control cultures, left column; shRNA-treated cultures, right column. Data are mean ± SD (n=3). **$p<0.01$ vs. controls. (e) Immunostaining for the differentiation markers; K10, loricrin, and involucrin staining of HaCaT 3D cell cultures. Control cultures, left column; shRNA-treated cultures, right column. Bar=50μm.

ShRNA-mediated silencing of B-Myb in NHKs inhibited epithelial thickning and decreased thickness in 3D culture

The 3D cultures of the control NHKs (Figure 4a, left column) and sh-B-Myb NHKs (Figure 4a, right column) were grown for 2-7 days. The mean thickness of the epithelial layers was measured after 2, 4, and 7 days of culture (Figure 4b). The cultures of shB-Myb NHKs became less thickened than the cultures of the control NHKs on days 2, 4, and 7 days. On days 2, 4 and 7, less Ki-67-positive cells were observed in the cultures of sh-B-Myb NHKs than that of the control NHKs (Figure 4c). The suppression of B-Myb resulted in decrease in thickness of undifferentiated layers which do not express the differentiation markers such as K10 and involucrin (Figure 5a).

ShRNA-mediated silencing of B-Myb in NHKs inhibited epithelial thickning and decreased thickness in 2D condition.

We also examined the effect of the change in B-Myb expression level on keratinocyte proliferation ability in 2D condition. Suppression of B-Myb resulted in a decrease in proliferation rate (Figure 5b).

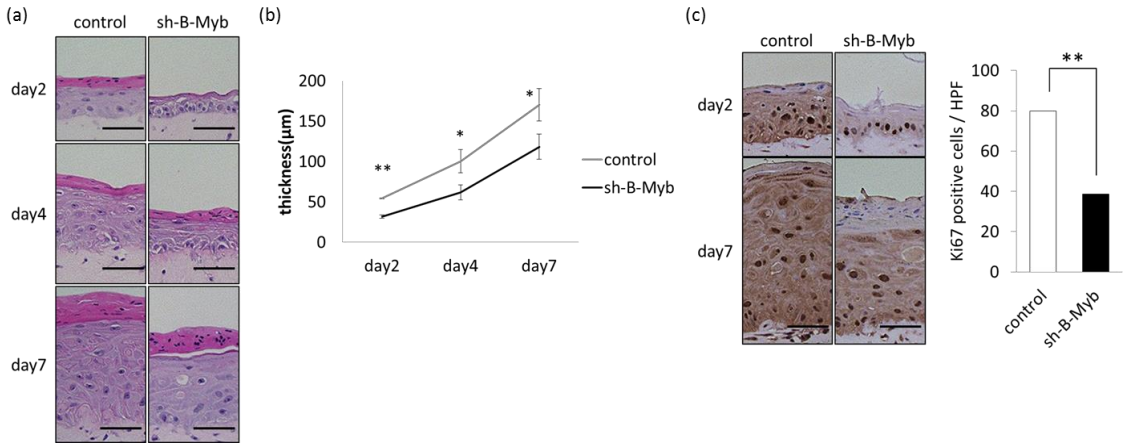

Figure 4

Effects of short hairpin RNA (shRNA)-mediated silencing of B-Myb expression on the morphology of normal human keratinocytes (NHKs).

(a) Hematoxylin and eosin (HE) staining of NHKs 3D cell cultures. Control cultures, left column; shRNA-treated cultures, right column. Silencing of B-Myb expression in NHKs inhibited growing thick. (b) Thickness of epidermoid layers. All values represent means ± SD (n=3). * $p<0.05$ vs. control. ** $p<0.01$ vs. control. (c) Immunostaining for Ki-67 staining of NHKs 3D cell cultures. Control cultures, left column; shRNA-treated cultures, right column. Data are mean ± SD (n=3). **$p<0.01$ vs. control.

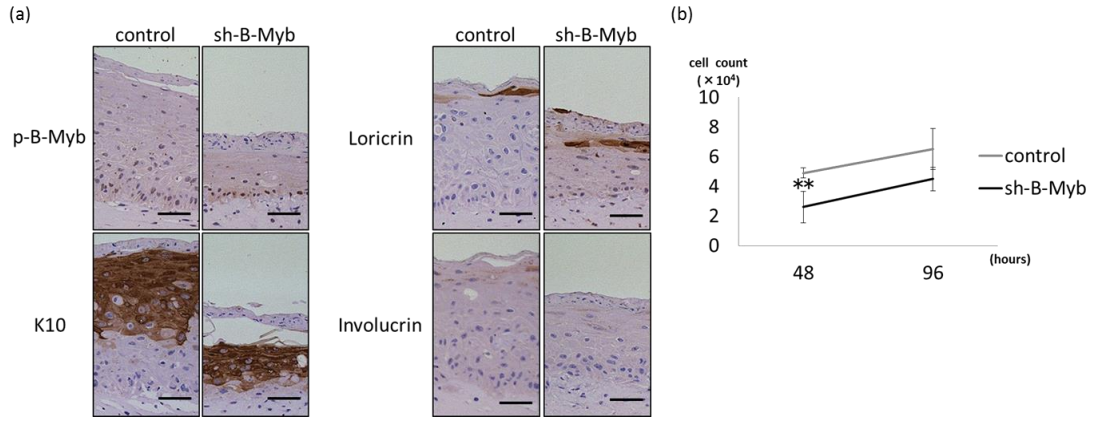

Figure 5

Effects of silencing of B-Myb expression about differentiation of normal human keratinocytes (NHKs) and proliferation rate.

(a) Immunostaining for the differentiation markers; K14, K10, loricrin, and involucrin staining of NHKs 3D cell cultures. Control cultures, left column; shRNA-treated cultures, right column. Bar=50μm. (b) Proliferation rate was assayed by cell counting under 2D condition. B-Myb silenced NHKs showed a decreased proliferation rate compared with control. **$p<0.01$ vs. control.

Overexpression of B-Myb in HaCaT cells has a positive effect on S and G2/M cell cycle regulatory factor expression

We analyzed the effects of B-Myb on expression of endogenous cell cycle and proliferation regulatory factors. Immunoblotting analysis and quantitative real-time PCR were performed to monitor the expression of cyclin A, CDK2, and CDK1, which are activators of cell proliferation and S phase and G2/M phase progression (Figure 6a and 6b). Overexpression of B-Myb in HaCaT cells resulted in upregulation of cyclin A, CDK2, and CDK1 expression at the mRNA level and overexpression of cyclin A and CDK1 on immunoblotting analysis. Also expression of phosphorylated B-Myb is upregulated on immunobloting analysis.

shRNA-mediated silencing of B-Myb has a negative effect on G2/M cell cycle regulatory factor expression at the mRNA level

We also analyzed the effects of B-Myb silencing on expression of endogenous cell cycle and proliferation regulatory factors. Immunoblotting analysis and quantitative real-time PCR were performed to monitor the expression of cyclin A, CDK2, and CDK1

(Figure 6c and 6d). Silencing of B-Myb in HaCaT cells resulted in downregulation of cyclin A, CDK2, and CDK1 at the mRNA level and decreased expression on immunoblotting analysis. Similar to HaCaT cells, silencing of B-Myb in NHKs induced downregulation of cyclin A, CDK2 and CDK1 in mRNA level (Figure 7).

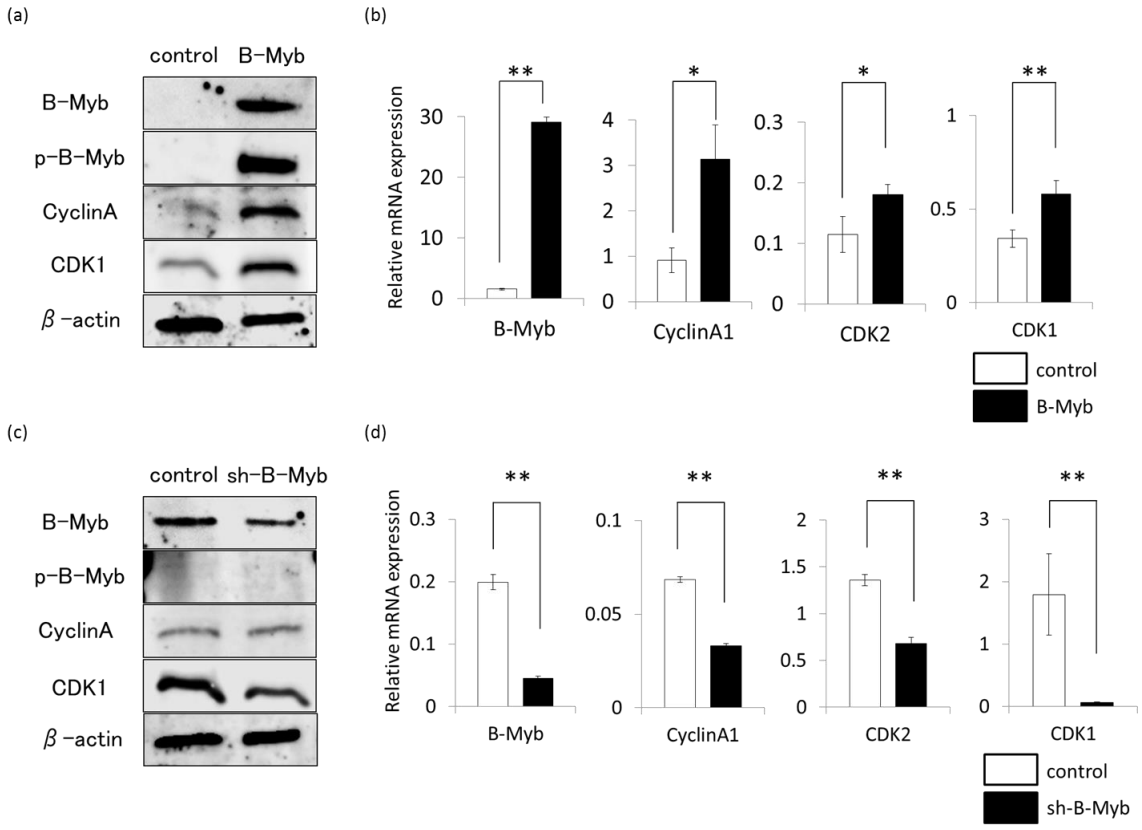

Figure 6

Effects of B-Myb overexpression and silencing on expression of cell cycle markers in HaCaT cells.

(a) Representative results of immunoblotting analysis of whole-cell lysates from HaCaT cells with specific antibodies for B-Myb, phosphorylated B-Myb, cyclin A, and CDK1. Control: Cells transfected with empty vector pAct. B-Myb: Cells transfected with

B-Myb expression vector pAct-B-Myb. (b) Total RNAs extracted from HaCaT cells were subjected to quantitative real-time PCR. Relative expression levels of B-Myb, cyclin A1, CDK2, and CDK1 mRNA. The expression levels were normalized relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression. * $P < 0.05$ vs. control, ** $P < 0.01$ vs. control. (c) Representative results of immunoblotting analysis of whole-cell lysates from HaCaT cells with specific antibodies for B-Myb, phosphorylated B-Myb, cyclin A, and CDK1. sh-B-Myb: Cells transfected with shRNA targeting B-Myb mRNA. Control: Cells transfected with non-target shRNA. (d) Total RNAs extracted from HaCaT cells were subjected to quantitative real-time PCR. Relative expression levels of B-Myb, cyclin A1, CDK2, and CDK1 mRNA. The expression levels were normalized relative to GAPDH mRNA expression. ** $P < 0.01$ vs. control.

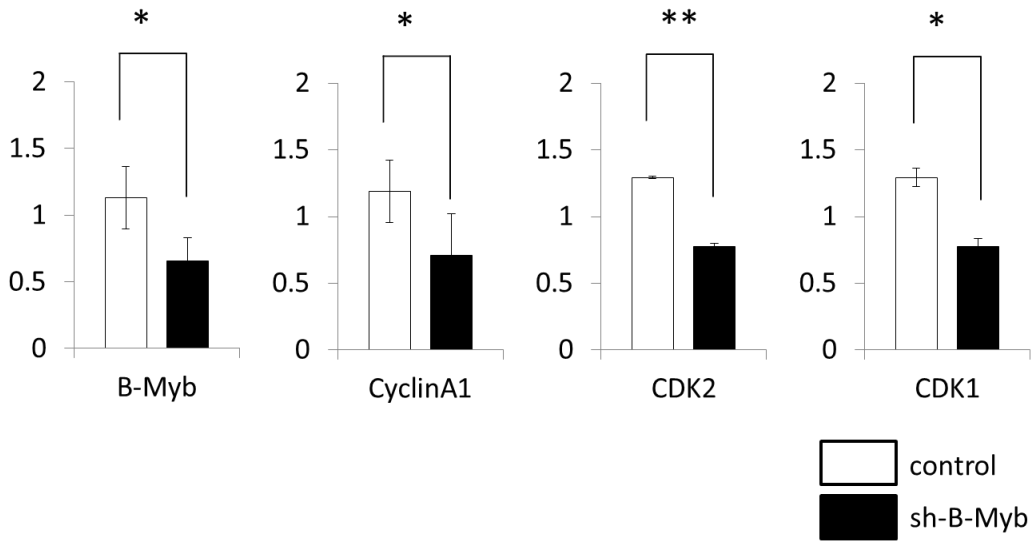

Figure 7

Effects of silencing of B-Myb expression on expression of cell cycle markers in NHKs.

Total RNAs extracted from NHKs were subjected to quantitative real-time PCR. Relative expression levels of B-Myb, cyclin A1, CDK2 and CDK1 mRNA. The expression levels were normalized relative to GAPDH mRNA expression. * $p<0.05$ vs. control, ** $p<0.01$ vs. control.

Discussion

Undifferentiated keratinocytes at the basal layer have high rates of proliferative activity, and these cells undergo cell cycle arrest and differentiation as they move up to the suprabasal layers. We showed that B-Myb, which is expressed specifically in undifferentiated keratinocytes, is downregulated during differentiation induced by the calcium switch. These observations support the reciprocity between proliferative capacity and differentiation.

The 3D cell culture of HaCaT cells used in the present study, in which cell monolayers are allowed to differentiate and undergo stratification in response to air exposure, is a suitable model in which to study the *in vitro* differentiation of keratinocytes (18). Using the 3D cell culture system, we showed that excess and constitutive expression of B-Myb in HaCaT cells resulted in arrest of cell differentiation, and that they retained the undifferentiated phenotypes of keratinocytes, including increased cell proliferation and suppression of differentiation markers, such as keratin 10, involucrin, and loricrin. Conversely, 3D cultures of sh-B-Myb HaCaT cells and sh-B-Myb NHKs showed decreased proliferation. These results suggest that B-Myb plays important roles in maintenance of keratinocytes in the undifferentiated state. The majority of previous

reports were consistent with the hypothesis that B-Myb activation is associated with cellular proliferation, as its expression can overcome G1 arrest in glioblastoma cells (21). Moreover, its expression appears to inhibit differentiation, as B-Myb was reported to be downregulated during colon epithelial cell maturation (16).

The B-Myb overexpression and suppression in 3D cell culture of HaCaT cells and NHKs indicated that B-Myb plays an important role in controlling cell proliferation. In addition, cell proliferation induced by overexpression of B-Myb suppressed epithelial keratinization and maintained the cells in the undifferentiated state. Experiments involving culture under 3D conditions indicated that excess B-Myb protein enhanced proliferation by upregulation of S and G2/M cell cycle accelerators, and the lack of B-Myb protein appeared to accelerate the differentiation of keratinocytes. Cyclin D1 quenches B-Myb transactivation until late G1 phase. From late G1 phase, B-Myb is phosphorylated and activated by cdk2 and cyclin A/E. In concert with E2F1-3, B-Myb regulates the expression of genes required for the G2/M phase of the cell cycle, such as cdc2, cyclin A2, and cyclin B1 (11). However, the role of B-Myb in cell cycle progression is dependent on cell type (17). The effects of B-Myb on keratinocytes are not yet clear. Overexpression of B-Myb in HaCaT cells resulted in upregulation of S phase proteins, such as cyclin A and CDK2, and G2/M phase proteins, such as cyclin A

and CDK1, at the mRNA and protein levels. Silencing of B-Myb in HaCaT cells and NHKs resulted in downregulation of cyclin A, CDK2, and CDK1 at the mRNA level. These results indicated that B-Myb promotes transcription of cyclin A, CDK2, and CDK1.

In conclusion, the findings of the present study suggested that B-Myb plays an important role in the suppression of keratinocyte differentiation and maintenance of the undifferentiated proliferative phenotype by modulating the expression levels of other cell cycle regulatory proteins, especially those expressed in the S and G2/M phases of the cell cycle.

Conflict of Interest

The authors have no conflicts of interest.

Acknowledgments

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