**Promoting effect of lactoferrin on barrier function and
epithelial differentiation of human keratinocytes**

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Complete List of Authors:	Uchida, Ryo; Tsukuba Daigaku, Graduate School of Life and Environmental Sciences ; National Agriculture and Food Research Organization, National Institute of Livestock and Grassland Science Aoki, Reiji; National Agriculture and Food Research Organization, National Institute of Livestock and Grassland Science Aoki-Yoshida, Ayako; The University of Tokyo, Graduate School of Agricultural and Life Sciences; National Agriculture and Food Research Organization, National Institute of Livestock and Grassland Science Tajima, Atsushi; University of Tsukuba, Faculty of Life and Environmental Sciences Takayama, Yoshiharu; National Agriculture and Food Research Organization, National Institute of Livestock and Grassland Science
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Promoting effect of lactoferrin on barrier function and epithelial differentiation of human keratinocytes

Ryo Uchida^{a,b}, Reiji Aoki^a, Ayako Aoki-Yoshida^{a,c}, Atsushi Tajima^b, and Yoshiharu Takayama^{a*}

a: Functional Biomolecules Research Group, National Institute of Livestock and Grassland Science, National Agriculture and Food Research Organization (NARO)

b: Graduate School of Life and Environmental Sciences, University of Tsukuba

c: Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo

*Corresponding author: Yoshiharu Takayama

Functional Biomolecules Research Group, National Institute of Livestock and Grassland Science, National Agriculture and Food Research Organization (NARO)

2 Ikenodai, Tsukuba, Ibaraki, 305-0901 Japan

Tel.: +81-29-838-8687, Fax: +81-29-838-8606, E-mail: takay@affrc.go.jp

Abbreviations

DMEM, Dulbecco's Modified Eagle's Medium; LRP-1, low-density lipoprotein receptor-related protein-1; MEK, mitogen-activated protein kinase kinase; PVDF, polyvinylidene difluoride; SREBP1, sterol regulatory element-binding protein-1; TER, transepithelial electrical resistance; MAPK, Mitogen-activated protein kinase; PI-3K Phosphoinositide 3-kinase; NHEK, normal human epidermal keratinocytes

Abstract

The purpose of this study was to elucidate the effects of bovine lactoferrin on keratinocyte differentiation and barrier function. Addition of bovine lactoferrin to differentiating HaCaT human keratinocytes led to increased transepithelial electrical resistance (TER), a marker of epithelial barrier function. This elevation was followed by upregulation of two differentiation markers, involucrin and filaggrin. The expression level of sterol regulatory element-binding protein-1 was also enhanced by bovine lactoferrin. The lactoferrin-induced upregulation of involucrin and filaggrin expression were confirmed in normal human epidermal keratinocytes (NHEK). Treatment with SB203580, a p38 mitogen-activated protein kinase (MAPK) α inhibitor, impaired the upregulation of involucrin and filaggrin expression in response to lactoferrin. The elevation of p38 MAPK phosphorylation was further enhanced by lactoferrin in the initial stage of differentiation of HaCaT keratinocytes. The findings suggest that bovine lactoferrin promotes epithelial differentiation by a p38 MAPK-dependent mechanism.

Keywords: involucrin, filaggrin, sterol regulatory element-binding protein-1, transepithelial electrical resistance, p38 mitogen-activated protein kinase

Introduction

The epidermis of the skin provides a protective barrier to the outside environment. Skin wound healing is a critical process to restore barrier function and prevent infection in response to disease or injury. Keratinocytes are a major cellular component of the epidermis and are responsible for restoring the epidermis after injury by a process termed re-epithelialization (Sivamani et al. 2007). The epidermis has a multilayered structure and consists of four progressively differentiated layers. During re-epithelialization, epidermal stem cells located in the basal layer of the epidermis continue to proliferate, whereas the newly produced daughter cells start to differentiate, migrating from the basal layer into the progressively differentiated layers, such as the spinous layer, granular layer, and stratum corneum. This involves formation of the cornified envelope, an insoluble complex of cross-linked proteins and lipids beneath the

1 plasma membrane of keratinocytes. The cornified envelope plays an important role in
2 maintaining the structural integrity and barrier function of the epidermis. Involucrin and
3 filaggrin are markers of the terminal differentiation of keratinocytes. Involucrin, one of
4 the major protein components of the cornified envelope, is present in the upper layers of
5 spinous cells and is maintained in keratinocytes of the granular layer (Eckert et al. 2004).
6 Filaggrin is a structural protein specifically found in the granular layer and stratum
7 corneum. Sterol regulatory element-binding protein-1 (SREBP1) is a transcription
8 factor that regulates the expression of enzymes involved in fatty acid and cholesterol
9 synthesis (Goldstein et al. 2006). Synthesis of fatty acids and cholesterol is required for
10 the barrier function of the epidermis. Earlier studies indicate that SREBP1 expression is
11 upregulated along with the differentiation of keratinocytes (Yokoyama et al. 2009). The
12 switch between keratinocyte growth and differentiation is tightly regulated. Loss of
13 proliferative potential is strictly associated with the differentiation of keratinocytes.

14 Lactoferrin is an iron-binding glycoprotein belonging to the transferrin family. It
15 can be detected in exocrine secretions of mammals such as milk, colostrum, tears, and
16 mucosal secretions. Lactoferrin contributes to host defense by its bactericidal and
17 immune-modulatory activities (Legrand et al. 2005; Actor et al. 2009). In addition,
18 recent studies indicate that topical administration of human lactoferrin increases the rate
19 of skin wound closure in normal and diabetic mice (Lyons et al. 2007; Engelmayer et al.
20 2008; Takayama and Aoki 2012). The promoting effect of human lactoferrin on wound
21 healing has been confirmed by clinical studies (Lyons et al. 2007). Lactoferrin promotes
22 the migration of epidermal keratinocytes and re-epithelialization of burn wounds in
23 swine (Tang et al. 2010; Takayama and Aoki 2012), suggesting that it promotes wound
24 healing by directly regulating epithelial keratinocytes.

25 We reported that bovine lactoferrin promotes the contractile activity of fibroblasts,
26 which play a critical role in wound healing by promoting wound contraction (Takayama
27 et al. 2003; Takayama and Aoki 2012). Furthermore, we found that bovine lactoferrin
28 promoted collagen and hyaluronan synthesis in human dermal fibroblasts, suggesting
29 that both human and bovine lactoferrin were effective for wound healing (Takayama
30 and Aoki 2012).

31 The purpose of this study was to investigate the effect of bovine lactoferrin on the
32 epithelial differentiation and barrier function of HaCaT keratinocytes. In this study, we

used HaCaT human keratinocytes as a model of epithelial differentiation of keratinocytes. Although HaCaT cells are immortalized cells, a similarity in the calcium-induced induction of differentiation markers and the proliferation rate in non-differentiating conditions was observed between HaCaT cells and normal human keratinocytes (Micallef et al. 2009).

Materials and methods

Materials

Bovine lactoferrin was obtained from Fonterra (Auckland, New Zealand). The iron saturation of bovine lactoferrin was less than 30%, <20 endotoxin units (EU)/mg, and >95% purity. HaCaT cells were provided by Cell Lines Service (Eppelheim, Germany). Normal human epidermal keratinocytes (NHEK) were provided by Lifeline Cell Technology (Frederick, MD, USA). High-glucose (4.5 g/ml) Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Sigma (St. Louis, MO, USA). HuMedia-KG2 medium was purchased from Kurabo (Osaka, Japan). Penicillin and streptomycin were obtained from Life Technologies (Carlsbad, CA, USA). CnT-07 keratinocyte growth medium and CnT-02 keratinocyte differentiation medium were purchased from CELLnTEC (Bern, Switzerland). Cell culture inserts were obtained from Corning (Corning, NY, USA). RIPA buffer was obtained from Nacalai Tesque (Kyoto, Japan). Anti-involucrin (clone SY5) and anti-SREBP1 (clone 2A4) monoclonal antibodies and an anti-filaggrin polyclonal antibody were obtained from Abcam (Cambridge, UK). An anti-occludin monoclonal antibody (clone OC-3F10) and anti-claudin-1 and anti-claudin-3 polyclonal antibodies were obtained from Invitrogen (Camarillo, CA, USA). Anti-p38 MAPK antibody, anti-phospho p38 MAPK antibody and phospho ERK1/2 antibody were obtained from Cell Signaling Technology (Danvers, MA, USA). An anti-GAPDH polyclonal antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Polyvinylidene difluoride (PVDF) membrane and enhanced chemiluminescence prime Western blotting detection reagent were purchased from GE Healthcare (Buckinghamshire, UK). A Millicell ERS-2 System was obtained from Millipore (Eschborn, Germany). MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] solution was obtained from Promega (Madison, WI, USA). PD98059 was

1 obtained from Cayman Chemical (Ann Arbor, MI, USA). LY294002 was purchased
2 from Jena Bioscience (Jena, Germany). SB203580 was obtained from Med Chem
3 Express (Princeton, NJ, USA).

4 **Cell culture**

5 HaCaT human keratinocytes were maintained in high-glucose DMEM
6 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml
7 streptomycin in an atmosphere of 5% CO₂ and 95% air at 37°C. NHEK were
8 maintained in HuMedia-KG2 medium in an atmosphere of 5% CO₂ and 95% air at 37°C.
9 For *in vitro* differentiation assays, subconfluent keratinocytes were grown in CnT-07
10 growth medium for 48 h. After reaching confluency, the medium was replaced with
11 CnT-02 differentiation medium supplemented with 1.8 mM CaCl₂.

12 **Transepithelial electrical resistance (TER) measurement**

13 A HaCaT keratinocyte suspension (1.8×10^5 cells) was seeded into a cell culture
14 insert with a pore size of 0.4 mm and a growth area of 0.6 cm², placed in a 24-well cell
15 culture plate. Medium was added to the cell culture plate such that the level was equal
16 to that inside the cell culture insert. Upon reaching confluency, HaCaT cells were
17 differentiated as described above. Following induction of epithelial differentiation, TER
18 was measured with a Millicell ERS-2 System every other day. Medium was replaced at
19 the same time. Data represent the mean of five independent measurements.

20 **Western blotting**

21 HaCaT cells and NHEK were harvested in RIPA buffer to yield cell lysates. Cell
22 lysates were resolved by 10% sodium dodecyl sulfate polyacrylamide gel
23 electrophoresis and electrically transferred to a PVDF membrane. Subsequently, the
24 membrane was treated with blocking reagent (Tris-buffered saline containing 0.1%
25 Tween-20 and 5% skimmed milk) for 2 h at room temperature. Blocked membranes
26 were incubated with primary antibodies (anti-involucrin (1:2000), anti-filaggrin
27 (1:1000), anti-SREBP1 (1:3000), anti-claudin-1 (1:2000), anti-claudin-3 (1:3000),
28 anti-occludin (1:3000), anti-p38 MAPK (1:2000), anti-phospho p38 MAPK (1:3000),
29 anti-phosphoERK1/2 (1:3000), anti-GAPDH (1:3000)), followed by incubation with a
30 horseradish peroxidase-conjugated secondary antibody (dilution ratio was 1:2000).
31 Immunoreactivity was detected using Western blotting detection reagents.

32 **Proliferation assay**

The proliferation of HaCaT cells in non-differentiating conditions was evaluated by quantification of formazan produced by reduction of MTS by living cells by using a kit (CellTiter 96® AQueous One Solution, Promega). HaCaT cells (100 µl) were plated in 96-well plates. MTS solution (20 µl) was added at the times indicated, and then cells were incubated at 37°C for 60 min. The amount of formazan was measured by colorimetric quantification (absorbance at 490 nm) using an iMark micro plate reader (Bio-Rad, Hercules, CA, USA). Results are expressed as means ± standard deviations.

Statistical analysis

Data were analyzed by IBM SPSS Statistics (version 21) using the Student's t-test. The limit for statistical significance was set at $P < 0.05$.

Results

Effect of lactoferrin on TER

We investigated whether lactoferrin can promote the barrier function of differentiating keratinocytes. After induction of differentiation, TER was measured every 2 days to evaluate the barrier function of HaCaT cells. Bovine lactoferrin (1–10 µM) was added to the differentiation medium at the onset of differentiation. A steady elevation of TER was observed in differentiating HaCaT cells in the absence of lactoferrin (Fig. 1). The elevation in TER was enhanced in lactoferrin-treated HaCaT cells, suggesting that lactoferrin improved the barrier function of differentiating keratinocytes (Fig. 1).

Effect of lactoferrin on the expression of differentiation markers

To evaluate the effects of lactoferrin on epithelial differentiation, we investigated the expression of involucrin and filaggrin by Western blotting at 5 days after induction of differentiation. When HaCaT cells were cultured in non-differentiating conditions (0.1 mM CaCl_2), a slight increase in involucrin and filaggrin expression was observed in response to lactoferrin (0.1–10 µM) (Fig. 2a). In HaCaT cells maintained in differentiating conditions (1.8 mM CaCl_2), expression of these differentiation markers was higher than in non-differentiating conditions at all lactoferrin concentrations ranging between 0.1 and 10 µM (Fig. 2a). The increased expression of involucrin and filaggrin was associated with the elevation of TER in differentiating conditions. This observation suggests that lactoferrin promotes the barrier function of keratinocytes by

1 promoting the differentiation and expression of cornified envelope components.

2 Next, we investigated the effect of lactoferrin on SREBP1 expression in HaCaT
3 cells (Fig. 2b). Lactoferrin-induced expression of SREBP1 was observed even in
4 non-differentiating conditions (0.1 mM CaCl_2). It was detectable in cells treated with
5 0.1 μM lactoferrin. When HaCaT cells were switched to differentiating conditions (1.8
6 mM CaCl_2), SREBP1 expression was detected even in the absence of lactoferrin.
7 SREBP1 expression was more pronounced in cells treated with 10 μM lactoferrin (Fig.
8 2b).

9 In the epidermis, skin barrier function is mainly mediated by cornified envelope
10 components. Tight junctions are another type of intracellular junction responsible for
11 skin barrier function (Tebbe et al. 2002). Therefore, we investigated the effect of
12 lactoferrin on expression of several tight junction components such as occludin,
13 claudin-1, and claudin-3 (Fig. 2c). Claudin-1 and -3 are the major protein species
14 present in the epidermis of healthy skin (Watson et al. 2007). In response to induction of
15 epithelial differentiation (1.8 mM CaCl_2), occludin expression was increased. However,
16 lactoferrin did not upregulate the expression of occludin or claudin-1 (Fig. 2c).
17 Interestingly, treatment of HaCaT cells with lactoferrin decreased expression of
18 claudin-3 in both differentiating and non-differentiating conditions in a dose-dependent
19 manner. These observations suggest that the promoting effects of bovine lactoferrin on
20 barrier function in keratinocytes are not mediated by elevation of the expression of tight
21 junction components.

22 **Effect of lactoferrin on the differentiation of normal human epidermal** 23 **keratinocytes**

24 The promoting effect of lactoferrin on epithelial differentiation was confirmed by
25 using normal human epidermal keratinocytes (NHEK). As HaCaT keratinocytes,
26 lactoferrin enhanced filaggrin and involucrin expression in NHEK maintained in
27 differentiating condition for 5 days (Fig. 2d). Lactoferrin-induced upregulation of
28 involucrin expression was also observed in NHEK maintained in non-differentiating
29 conditions (0.1 mM CaCl_2).

30 **Effect of lactoferrin on the proliferation of HaCaT keratinocytes**

31 It is reported that loss of proliferative potential is associated with the
32 differentiation of keratinocytes. We performed the MTS colorimetric assay to evaluate

the effect of bovine lactoferrin on HaCaT cell proliferation. The proliferation rate did not differ in the presence or absence of bovine lactoferrin until day 2, whereas an inhibitory effect of bovine lactoferrin on proliferation was observed at day 3 (Fig. 3). The effect of bovine lactoferrin was dose-dependent at concentrations ranging between 1 and 10 μ M.

Effect of signaling inhibitors on lactoferrin-induced involucrin and filaggrin expression

Next, we investigated the signaling molecules required for lactoferrin-induced epithelial differentiation of HaCaT keratinocytes. Treatment with SB203580, a p38 mitogen-activated protein kinase (MAPK) α inhibitor, impaired lactoferrin-induced upregulation of involucrin and filaggrin expression, whereas an inhibitory effect of LY294002, a phosphoinositide 3-kinase (PI-3K) inhibitor, was observed only on involucrin expression (Fig. 4a). PD98059, a MAPK kinase (MEK)/ERK1/2 inhibitor, had no effect on involucrin or filaggrin expression. Inhibition of p38MAPK δ is toxic to HaCaT keratinocytes; therefore, the effect of p38MAPK δ inhibitors on involucrin and filaggrin expression was not carried out in the present study.

Effect of lactoferrin in p38MAPK in differentiating keratinocytes

To confirm the involvement of p38MAPK on lactoferrin-enhanced epithelial differentiation, we investigated the time course of p38MAPK α and ERK1/2 phosphorylation in differentiating HaCaT cells (Fig. 4b). Un-differentiated cells exhibited little p38MAPK α phosphorylation, along with increased phosphorylation at day 4, followed by a return to baseline levels at day 6. In the presence of lactoferrin, enhanced p38MAPK α phosphorylation was observed in the initial stage of epithelial differentiation (day 2). In contrast, ERK1/2 phosphorylation was observed in un-differentiated HaCaT keratinocytes. Induction of epithelial differentiation resulted in suppression of ERK1/2 phosphorylation in the presence or absence of lactoferrin. These observations suggest that lactoferrin promotes epithelial differentiation by a p38MAPK α -dependent mechanism.

Discussion

The results of this study indicate that bovine lactoferrin promotes the elevation of

1 TER in differentiating keratinocytes (Fig. 1). TER reflects the permeability of cell
2 layers to ions, with a higher TER indicating a lower ionic permeability (Francis et al.
3 1999). Therefore, our results suggested that bovine lactoferrin promotes the barrier
4 function of keratinocytes. In the epidermis, the barrier function is mainly mediated by
5 the cornified layer. Lactoferrin-induced elevation of TER was associated with increased
6 expression of cornified envelope components such as involucrin and filaggrin (Fig. 2a).
7 In differentiating (high calcium) conditions, expression of involucrin and filaggrin was
8 observed even when the lactoferrin concentration was relatively low (0.1 μ M). In
9 non-differentiating conditions (low calcium), expression of the differentiation markers
10 was low. This observation indicates that expression of the differentiation markers is
11 tightly regulated by extracellular calcium concentrations. The role of lactoferrin in
12 keratinocyte differentiation may reinforce the calcium-induced signaling pathway
13 required for keratinocyte differentiation.

14 Lactoferrin-induced expression of involucrin was blocked by SB203580, an
15 inhibitor of p38MAPK α (Fig. 4a). Many studies of the role of p38MAPK in
16 keratinocyte differentiation focus on p38MAPK δ (Eckert et al. 2003). Many agents that
17 promote involucrin gene expression increase p38MAPK δ kinase activity in
18 keratinocytes (Eckert et al. 2003; Eckert et al. 2004; Cuenda et al. 2007). In addition,
19 p38MAPK α also plays an important role in involucrin gene expression and keratinocyte
20 differentiation. Inhibition of p38MAPK α impairs involucrin gene expression induced by
21 treatment with methyl- β -cyclodextrin or ectopic expression of several MEKs in
22 keratinocytes (Cuenda et al. 2007; Dashti et al. 2001; Jans et al. 2004). Although the
23 mechanisms underlying lactoferrin-induced activation of p38MAPK α have not been
24 elucidated, lactoferrin is likely to enhance involucrin expression by a
25 p38MAPK α -dependent mechanism. In addition, lactoferrin-induced enhancement of
26 involucrin expression was blocked by LY294002, a PI-3K inhibitor, suggesting that the
27 promoting effect of bovine lactoferrin on keratinocyte differentiation is also dependent
28 on the PI-3K signaling pathway (Fig. 4a). This result is consistent with previous studies
29 reporting that the activation of PI-3K in keratinocytes promotes growth arrest and
30 differentiation. Activation of PI-3K is observed at early stages of mouse keratinocyte
31 differentiation both in culture and in the intact epidermis *in vivo* (Calautti et al. 2005).
32 Inhibition of PI-3K blocks calcium-induced expression of involucrin in keratinocytes

(Calautti et al. 2005; Xie et al. 2005). In this study, expression of SREBP1 was also enhanced by treatment with lactoferrin (Fig. 2b). A previous report showed a role of PI-3K in SREBP1 expression in HaCaT keratinocytes (Zhou et al. 2012). However, we noted that lactoferrin-induced expression of SREBP1 was observed even in non-differentiating (low calcium) conditions (Fig. 2b), suggesting that the mechanism underlying lactoferrin-induced enhancement of SREBP1 expression is distinct from that of involucrin and filaggrin. Further studies are required to elucidate the mechanisms by which lactoferrin induces SREBP1.

In epidermis, involucrin is a major component of the cornified envelope, a structure synthesized at late stages of keratinocyte differentiation. Physical crosslinking of involucrin to other components of cornified envelope contributes to sustain epidermal barrier integrity. Lactoferrin-induced enhancement of involucrin expression suggests that the function of lactoferrin is mainly mediated by reinforcement of the cornified envelope. We did not observe a promoting effect of bovine lactoferrin on the expression of tight junction components (occludin, claudin-1). Claudin-3 expression was decreased in response to lactoferrin (Fig. 2c). Whereas epithelial barrier function is mainly sustained by cornified layer of epidermis, claudin-3 was only present in granular layer in epidermis (Watson et al. 2007). Therefore, this reduction is not likely to affect epithelial barrier function in HaCaT keratinocytes.

Lactoferrin exerts its biological activity by binding to its receptor(s) (Suzuki et al. 2005). Tang *et al.* reported that the promoting effect of human lactoferrin on the migration of keratinocytes is dependent on low-density lipoprotein receptor-related protein-1 (LRP-1) (Tang et al. 2010). LRP-1 acts as a lactoferrin receptor in fibroblasts, osteoblasts, and adipocytes that converts the lactoferrin-binding signal into activation of the ERK1/2 signaling pathway (Takayama et al. 2003; Grey et al. 2004; Ikoma-Seki et al. 2015). In this study, lactoferrin-induced involucrin expression was not dependent on ERK1/2 (Fig. 4). ERK1/2 is reportedly a negative regulator of involucrin expression (Eckert et al. 2004). Therefore, a receptor(s) other than LRP-1 may be involved in the promoting effect of lactoferrin on keratinocyte differentiation.

Defects in keratinocyte differentiation and skin barrier function cause inflammatory skin diseases such as atopic dermatitis (Cork et al. 2009). The results of this study suggest that lactoferrin contributes to host defense by increasing keratinocyte

1 barrier function.

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1 Figure legends

2 **Fig. 1.** Effect of bovine lactoferrin on the transepithelial electrical resistance (TER) of
3 differentiating HaCaT cells. After induction of epithelial differentiation, HaCaT cells
4 were mock-treated (open circles) or treated with lactoferrin (1 μ M: filled circles, 5 μ M:
5 open triangles, 10 μ M: filled squares) for the indicated amount of time. Error bars
6 represent the mean $\pm$ standard deviation. n=3 in five independent experiments.
7 **P<0.01 and *P<0.05 versus mock-treated cells.

8

9 **Fig. 2.** Effect of bovine lactoferrin on epithelial differentiation of keratinocytes. (a)
10 Expression of filaggrin and involucrin in HaCaT keratinocytes. Confluent HaCaT cells
11 were allowed to differentiate (1.8 mM CaCl_2) for 5 days or were cultured in
12 non-differentiating conditions (0.1 mM CaCl_2) for the same amount of time. The
13 indicated concentrations of bovine lactoferrin were added to the culture media. (b)
14 Expression of sterol regulatory element-binding protein-1 (SREBP1). (c) Expression of
15 tight junction proteins. GAPDH was used as a loading control. (d) Expression of
16 filaggrin and involucrin in normal human epidermal keratinocytes. Experimental
17 procedure is same as for HaCaT keratinocytes.

18

19 **Fig. 3.** Effect of bovine lactoferrin on the proliferation of HaCaT keratinocytes. HaCaT
20 cells were mock-treated or treated with bovine lactoferrin (1–10 μ M) for the indicated
21 amount of time. Relative numbers of HaCaT cells were assessed by measuring
22 absorbance at 490 nm, which corresponds to formazan production by living cells. Error
23 bars represent the mean $\pm$ standard deviation. **P<0.01 versus mock-treated cells.

24

25 **Fig. 4.** The involvement of the p38MAPK signaling pathways in lactoferrin-induced
26 differentiation of HaCaT keratinocytes. (a) HaCaT cells were differentiated in the
27 presence or absence of 10 μ M bovine lactoferrin for 5 days. PD98059 (40 μ M),
28 SB203580 (10 μ M), or LY294002 (10 μ M) were added at the same time. Cell
29 differentiation was evaluated by the expression levels of involucrin and filaggrin.
30 GAPDH was used as a loading control. (b) Effect of lactoferrin on phosphorylation of
31 p38MAPK and ERK1/2 in differentiating HaCaT cells. HaCaT cells were differentiated
32 in the presence or absence of 10 μ M bovine lactoferrin for the indicated times.

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