Lactoferrin and lactoferrin-sophorolipids-assembly can be internalized by dermal fibroblasts and regulate gene expression

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Lactoferrin and lactoferrin-sophorolipids-assembly can be internalized by
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Abstract: Lactoferrin (Lf) is an iron-binding multifunctional protein, mainly present in external secretions. Lf is known to penetrate skin and may thus exert its multiple functions in skin. Sophorolipids (SLs) are glycolipid biosurfactants, which have been shown to enhance absorption of commercial bovine Lf (CbLf) in model skin via forming an assembly with CbLf. In the present study, uptake and post-internalization localization of bovine Lf (bLf), CbLf, and human Lf (hLf) with or without forming assemblies with SLs in human dermal fibroblasts (HDFn) were determined using ^{125}I -labeled Lfs and confocal microscopy, respectively. Our results show that all three Lfs were internalized by HDFn; although SLs did not significantly affect uptake of Lfs, it changed Lf localization by accumulating Lfs to the perinuclear region. Furthermore, microarrays were used to investigate transcriptional profiling in HDFn in response to CbLf, SLs, or CbLf-SLs-assembly treatments. Transcriptome profiling indicates that CbLf may play roles in protection of skin from oxidative stress, immunomodulatory activities, and enhancement of wound healing. The assembly had similar effects but dramatically modulated transcription of some genes. SLs alone modified signaling pathways related to lipid metabolism, as well as synthesis of sex hormones and vitamins. Thus, CbLf may exert beneficial effects on skin, and these effects may be modulated by SLs.

Key words: lactoferrin, sophorolipids, dermal fibroblasts, internalization, gene expression

Introduction

Lactoferrin (Lf) is an iron-binding protein mainly present in external secretions such as breast milk, saliva, tears, bile, and pancreatic fluids (Lönnerdal 2009). It is a multifunctional protein involved in a wide range of activities, such as immunomodulation, and promotion of cell proliferation (Vogel 2012). Effects of Lf on skin comprise promotion of skin wound healing (Takayama and Aoki 2012), improvement of inflammatory lesions of *acne vulgaris* (Kim et al. 2010; Mueller et al. 2011), up-regulation of proliferation of dermal fibroblasts and keratinocytes (Engelmayer et al. 2008; Tang et al. 2010), and attenuation of UV-induced photo damage (Murata et al. 2014). Lf is known to penetrate skin (Ishii et al. 2012) and has high water solubility. Therefore, it may be feasible to add Lf to water-based cosmetics. In general, Lf exerts its multiple functions via two mechanisms; 1) working as a transcription factor to transactivate gene expression (He and Furmanski 1995), or 2) binding to a specific receptor to initiate signaling pathways (Jiang and Lönnerdal 2014). Commercial bovine Lf (CbLf) is available, but its bioactivities may not be the same as those of bLf isolated in the laboratory due to different processing conditions. Human Lf (hLf) and bovine Lf (bLf) may differ in functions as hLf and bLf only share 69% amino acid sequence identity (Lönnerdal et al. 2011). Iron-free Lf (apo-Lf) and iron-saturated Lf (holo-Lf) may also function diversely because they have markedly different conformations (Grossmann et al. 1992). For this reason, apo- and holo- forms of several Lfs were investigated in the present study.

Sophorolipids (SLs) are glycolipid biosurfactants, generated by fermentation of a number of non-pathogenic yeasts. Each SL is consisted of a dimeric sugar, a sophorose,

and a long chain hydroxyl fatty acid with 16 or 18 carbon atoms. SLs can form a vesicle-like structure to promote transport of substances into cells through size reduction and encapsulation (Imura et al. 2014; Zhou et al. 2004). SLs produced by *Starmerella bombicola* have been shown to enhance CbLf absorption by model skin up to 1.7-fold via forming an assembly with CbLf. Moreover, SLs significantly regulated gene expression upon the CbLf treatment (Ishii et al. 2012). In addition to being biodegradable surfactants, SLs possess skin compatibility, anti-inflammation (Bluth et al. 2006), and antimicrobial activities (Azim et al. 2006; Diaz De Rienzo et al. 2016; Solaiman et al. 2015), as well as good moisturizing and depigmenting properties (Lundov et al. 2009). Therefore, SLs have been applied in various cosmetic formulations (Morya et al. 2013; Van Bogaert et al. 2007; Varvaresou and Iakovou 2015).

The objectives of the present study were to examine uptake and post-internalization localization of apo- and holo-Lfs, as well as effects of Lfs and SLs on dermal fibroblasts, and thus to provide preliminary data and a scientific basis for addition of Lf and SLs to cosmetic products. To address the objectives, we examined whether hLf, bLf, and CbLf were internalized by human dermal fibroblasts (HDFn) by using I-125 labeled Lfs and the post-internalization localization of Lfs in HDFn by confocal microscopy. The microarray is a powerful tool to identify genes simultaneously relevant to various treatments (Papp et al. 2012), and we therefore used microarrays to characterize global transcription profiles of CbLf-, SLs-, and CbLf-SLs-assembly-treated human dermal fibroblasts (HDFn).

Materials and methods

Preparation of hLf and bLf

Human-Lf (hLf) and bovine Lf (bLf) were isolated from milk as described previously (Lönnerdal et al. 2011). To examine the purity and concentration of Lf in each eluted fraction, each sample (10 μ L) was subjected to SDS-PAGE and gels were stained with Coomassie Brilliant Blue R-250 (Sigma, St Louis, MO). Based on the gel results, only Lf isolated from fraction 2 with an elution buffer containing the higher concentration of NaCl (1.0 mol/L) was used for subsequent experiments. CbLf (~15% iron saturation) was purchased from Morinaga Milk Industry Co, Ltd. (Tokyo, Japan). Iron-free Lf (Apo-Lf) and iron saturated Lf (holo-Lf) were prepared as described previously (Lopez et al. 2008; van Berkel et al. 1995).

Preparation of SLs and Lf-SLs-assemblies

SLs were produced by *Starmerella bombicola* NBRC10243. Stock solutions of SLs and Lf-SLs-assemblies were prepared using methods described previously (Ishii et al. 2012).

Cell culture

Human dermal fibroblasts (HDFn, Cascade Biologics, Portland, OR) were maintained in a humidified incubator at 37°C under an atmosphere of 5% CO₂ in Medium 106 supplemented with Low Serum Growth Supplement (LSGS, Cascade Biologics, Portland, OR). The medium was changed every other day, and cells between passages 5-10 were used in the present study.

Lf uptake

All Lfs were labeled with ^{125}I (GE Healthcare, Waukesha, WI) using the Iodogen (Pierce, Rockford, IL) method. HDFn were seeded on 24-well plates (2.5×10^3 cells/cm²). After cells were cultured for 10 days, D10 HDFn on 24-well plates were incubated in supplement-free medium (SFM) containing ^{125}I -labeled Lf samples (100 µg/mL), or Lf-SLs-assembly (100 µg/mL Lf and 10 µg/mL SLs) for 30 min at 37°C. Cells were rinsed with ice-cold PBS 3 times and then liquefied with NaOH (1 mol/L). After cells were solubilized, cell-associated radioactivity was quantified in a gamma counter (Packard Minaxi γ; Meriden, CT). Non-specific binding was assessed by incubation with unlabeled Lf (100-fold molar excess) and subtracted from total uptake, resulting in specific uptake.

Indirect fluorescence

HDFn were seeded and grown on Lab-TekII chambered slides (Nalge Nunc International, Naperville, IL) overnight. After cells were treated with Lfs (100 µg/mL), Lf assemblies (100 µg/mL), or SLs (10 µg/mL) in SFM for 30 min at 37°C, cells were rinsed with PBS, fixed with phosphate-buffered paraformaldehyde (4%, 0.4 mL/well) for 10 min at room temperature, and then permeabilized with Triton X-100 (0.2%) in PBS for 10 min. Cells were subsequently blocked with blocking buffer (5% heat-inactivated rabbit serum and 1% BSA in PBS, 0.5 mL/well) for 20 min. After the blocking buffer was removed, cells were rinsed with PBS. Lf was subsequently probed with rabbit anti-hLf (2 µg/mL, Abcam, Cambridge, MA), or goat anti-bLf (2 µg/mL, Bethyl Laboratories, Montgomery, TX), and then Alexa 488-conjugated-anti-rabbit or Alexa 488-conjugated-

anti-goat IgG (1 µg/mL; Molecular Probes, Eugene, OR) in blocking buffer for 30 min. The nucleus was stained with TOPO-3 (1:1000 in blocking buffer, Molecular Probe, Eugene, Oregon) for 30 min. After several rinses, coverslips were mounted with ProLong Gold Antifade Reagent (Invitrogen Molecular Probes) and sealed with nail polish. A confocal laser scanning microscope (FV1000, Olympus America, Inc., Melville, NY) was used to perform immunofluorescence imaging, and image analysis software systems (Olympus America, Inc.) were applied to analyze the images. The density of Lf fluorescence and the ratio of perinuclear to total internalized Lf in 20 cells from four slides for each treatment group were measured by using analytical tools from Image J (<http://imagej.nih.gov/ij/>). The perinuclear area was defined as the cytoplasmic area encircling the nucleus and having a radius around 10 µm.

RNA extraction and array hybridization

HDFn (6-well plate, $2.5 \times 10^3/\text{cm}^2$) were grown for 24 h and then treated with CbLf (100 µg/mL), SLs (10 µg/mL), or CbLf-SLs-assembly for 24 h. RNA was subsequently extracted with Trizol (Invitrogen), and then purified by the RNease kit (Qiagen) according to the manufacturer's instructions. Measurement of RNA yield was performed using a NanoDrop 1000A Spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and RNA quality was evaluated by Bioanalyzer RNA Nano Chips (Agilent Technologies, Inc., Santa Clara, CA) following the manufacturer's procedure. Total RNA samples were amplified and labeled with biotinylated nucleotides using a kit (Ambion, TotalPrep -96 RNA Amplification Kit). Bioanalyzer analysis was then carried out to verify if cRNA was at the expected the 1.2 kb average size before applying to

beadchips (HumanHT-12 v.4.0, Illumina, San Diego). Beadchips were scanned with the Illumina iScan using standard conditions.

Microarray data analysis

GenomeStudio (Illumina) was utilized for microarray data analysis and quantile normalization was carried out to normalize the microarray data. Only probe sets with adjusted p-value < 0.05 were selected for subsequent analysis and totally 13716 genes were analyzed. The differentially expressed genes were selected following two criteria: fold-change >1.5 for up-regulation, and fold-change < 0.5 for down-regulation. Samples were clustered with a centroid linkage method on the horizontal axis, and the lengths of the branches were measure by a Euclidean algorithm. Pathway analysis was conducted using IPA (Ingenuity® Systems, www.ingenuity.com) and the Core analysis included in IPA was chosen to interpret data in the context of biological processes, pathways, and networks. A p-value was calculated using the right-tailed Fishers exact test for each canonical pathway and p-values less than 0.05 were considered significantly relevant. Heatmapper (<http://www.heatmapper.ca/>) was used to generate the heatmap.

The accession number

Microarray results have been deposited in the NCBI Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) and are available through the accession number (GSE84144).

Quantitative real-time PCR

RNA (1 µg) was reverse-transcribed to cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. Gene-specific primers are listed in table 1. Real-time polymerase chain reaction (RT-PCR) was performed on the cDNA reaction mixture (2 µL) and SYBR Green (Thermo Scientific) using the iCycler real-time PCR system (Bio-Rad). The cycling parameters were 95°C 15 min and 40 cycles including 95°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec. Linearity of the dissociation curve was analyzed using the iCycler software and the mean cycle time of the linear part of the curve was designated as Ct. Each sample was analyzed in sextuplicate and normalized to GAPDH using the following equation: fold change = $2^{(Ct_{\text{Gene}} - Ct_{\text{GAPDH}})}$. Values are shown as mean fold change ± standard deviation, relative to the non-treated control (set to 1).

Statistical analysis

Data represent means ± standard deviations from at least 3 independent experiments. Comparisons between the treatment and control were conducted using one-way ANOVA (Prism Graph Pad, Berkeley, CA). P<0.05 was considered to be statistically significant.

Results

Apo- and holo-Lf are internalized by HDFn

To determine if bLf and CbLf were internalized by HDFn, they were labeled with ¹²⁵I by using the Iodogen method. Based on our pilot study, the Lf receptor (LfR), originally isolated from the human small intestine (Suzuki et al. 2001), and LRP1, a

functional LfR in fibroblasts (Takayama et al. 2003), displayed peak expression at day 10 (data not shown). D10 HDFn were therefore used for uptake experiments. As shown in Fig. 1, CbLf and bLf were internalized by HDFn, and no differences were found in uptake between HDFn incubated with Lfs and Lf-SLs-assemblies. As a comparison, we included hLf and the results were similar to those for the two bovine Lfs. All apo-forms of Lf showed significantly higher uptake than holo-forms of Lf, suggesting that conformation differences influence internalization of Lf by HDFn. Apo-bLf exhibited the highest uptake among all Lfs.

Indirect fluorescence detection was next conducted to examine if SLs influence intracellular localization of Lfs after internalization. The results from confocal microscopy confirmed that all Lfs were internalized by HDFn (Fig. 2). The post-internalization localization of all Lfs was altered by SLs as significant amounts of hLf, bLf, and CbLf were localized in the perinuclear area upon treatments with Lf-SLs-assemblies. Localization of Lfs was not affected by iron saturation for any of the Lfs. Although the major part of Lfs was localized in the cytoplasm, co-localization of Lf and the nucleus was observed for all treatments, suggesting that all the Lfs are capable to enter the nucleus of HDFn and thereby exert multiple functions.

Microarray analysis of HDFn treated with CbLf, SLs, and CbLf-SLs-assembly

Since any practical application of Lf will be dependent upon commercially available bLf, effects of CbLf, SLs, or CbLf-SLs-assembly on HDFn were investigated by microarray analysis. According to the cluster analysis (Fig. 3) and heatmap (Fig. 4), transcription of genes was differentially and significantly regulated by CbLf, SLs, and

221 CbLf-SLs-assembly. The cluster analysis of the entire genome indicates that the three
222 biological replicates were consistent, and that CbLf and CbLf-SLs-assembly displayed
223 similar effects. As shown in Fig. 3, CbLf- and CbLf-SLs-assembly-treated samples
224 formed distinct clusters, separately from the control and SLs-treated samples, indicating
225 that CbLf and CbLf-SLs-assembly significantly altered transcriptional profiles of HDFn,
226 and that SLs-treated HDFn exhibited the weakest transcriptional response. Consistently,
227 the heatmap generated by markedly modified genes also showed that CbLf and CbLf-
228 SLs-assembly significantly changed gene transcription of HDFn, and similar effects on
229 HDFn were found in presence of CbLf or CbLf-SLs-assembly (Fig.4).

230 As shown by a Venn diagram (Fig. 5), CbLf, SLs, and CbLf-SLs-assembly
231 significantly regulated gene transcription in HDFn. Total genes modulated by CbLf, SL,
232 and CbLf-SLs-assembly were 1520, 100, and 1472, respectively. 1263 genes, about
233 86% of genes changed by CbLf were modified by both CbLf and CbLf-SLs-assembly,
234 indicating that SLs forming an assembly with CbLf only slightly change effects of CbLf
235 on transcription profiling. Although SLs alone significantly stimulated transcription of
236 some genes, the effects were much weaker than those initiated by CbLf and CbLf-SLs-
237 assembly.

238 **Validation of microarray results by Real-time PCR**

239 To verify results from the microarray analysis, six genes (FABP3, COL7A1,
240 CXCL6, PLIN2, CPA4, and EDN1) were selected for real-time PCR analysis in terms of
241 expression intensity and various patterns of gene expression regulated by CbLf, SLs, or
242 CbLf-SLs-assembly (Fig. 6). For example, the FABP3 gene was selected because this

gene was significantly modified by all three treatments, and the EDN1 gene was chosen because only SLs had a significant effect on transcription of this gene. The results from these six genes were consistent with results from the microarrays and the R^2 of association analysis was higher than 0.95 (Fig. 6G), indicating that the two data sets were strongly correlated. Thus, results from the microarray assays were validated.

Effects of CbLf and CbLf-SLs-assembly on proliferation of HDFn

CbLf and CbLf-SLs-assembly significantly stimulated transcription of genes involved in cellular proliferation. As shown in Supplemental table 1, these genes comprised kinases, transcription factors, cell cycle regulators, growth factors, and components of the extracellular matrix. CbLf and the assembly showed similar effects, except for few genes on which CbLf had stronger effects than the assembly. Since SLs did not significantly modulate transcription of any of these genes listed in the Table, only results for CbLf and CbLf-SLs-assembly are shown.

According to pathway analysis ($p < 0.05$), a number of signaling pathways induced by CbLf and the assembly are involved in regulating transcription of proliferation related genes (Supplemental table 3). PI3K/AKT, PDGF, and IGF-1 pathways were three major signaling pathways by which CbLf and CbLf-SLs-assembly increased proliferation of HDFn. In addition, CbLf-SLs-assembly activated VEGF signaling to exert its proliferative effects on HDFn.

Effects of CbLf and CbLf-SLs-assembly on transcription of genes involved in migration of HDFn

In agreement with enhancing effects of Lfs on migration of dermal fibroblasts (Engelmayer et al. 2008; Francis et al. 2011), both CbLf and CbLf-SLs-assembly significantly promoted transcription of genes mediating cell migration (Supplemental table 1), including growth factors, extracellular matrix and related enzymes, suggesting that CbLf and CbLf-SLs-assembly may play a role in enhancement of cell migration.

CbLf and CbLf-SLs-assembly up-regulated expression of genes involved in generation of the extracellular matrix

Our results show that CbLf and CbLf-SLs-assembly markedly enhanced transcription of components of the extracellular matrix (ECM) (Supplemental Table 1), which is consistent with previous reports showing that CbLf promoted secretion of hyaluronan in human normal dermal fibroblasts (Saito et al. 2011; Takayama et al. 2003). Compared with effects regulated by CbLf, the assembly had stronger or weaker effects on transcription of some genes, such as TIMP, PLOD2, and VCAN. Expression of collagen I, the most abundant collagen in skin, was significantly up-regulated by CbLf. Besides proteins of the ECM, CbLf and CbLf-SLs-assembly also enhanced transcription of enzymes required for construction of ECM, and increased transcription of inhibitors for matrix metalloproteinases (MMPs), which degrade collagens. Put together, CbLf and CbLf-SLs-assembly up-regulated ECM synthesis via promotion of synthesis of ECM components and their required enzymes, as well as up-regulation of MMP inhibitors.

CbLf and CbLf-SLs-assembly promoted transcription of genes involved in resistance against oxidative stress

CbLf and CbLf-SLs-assembly significantly increased the potential for oxidative stress resistance in dermal fibroblasts via stimulating transcription of genes (Supplementary table 1) involved in removal of reactive oxygen species (ROS), particularly superoxide dismutase (SOD). Two signaling pathways modified were found to be involved in antioxidant reactions, namely NRF2 oxidative stress response, and the antioxidant action of vitamin C (Supplementary table 3).

Effects of CbLf and CbLf-SLs-assembly on expression of genes involved in Immunomodulatory activities

CbLf and CbLf-SLs-assembly considerably stimulated transcription of genes related to immunomodulatory effects. As shown in Supplementary table 1, expression of genes for cytokines, chemokines, and kinases were dramatically increased. The immunomodulatory effects were mediated by several signaling pathways (Supplementary table 3). Dominant signaling pathways including IL-6, iNOS, Tweak, IL-8, and IL-17A signaling mediated effects triggered by both CbLf and CbLf-SLs-assembly.

Effects on lipid metabolism and other effects of SLs on HDFn

CbLf, CbLf-SLs-assembly, and SLs promoted cholesterol biosynthesis of HDFn (Supplementary tables 2&4). Although CbLf, assembly, and SLs activated similar signaling pathways to enhance cholesterol biosynthesis, the treatment with SLs alone exhibited the highest efficacy. In addition to the effect on lipid metabolism, SLs enhanced biosynthesis of estrogen, androgen, and vitamin A &D (Supplementary table 5).

308 **Pathway analysis summary**

309 CbLf and CbLf-SLs-assembly significantly regulated gene transcription profiling
310 of HDFn. A large number of genes involved in diverse biological processes are up-
311 regulated in the transcriptional response. Based on the pathway analysis, CbLf and
312 CbLf-SLs-assembly play critical roles in cellular functions (Supplementary table 6). The
313 major functions regulated by CbLf and CbLf-SLs-assembly consisted of positive
314 regulation of cellular assembly and organization, as well as cell cycle progression. The
315 main effects of SLs on dermal fibroblasts comprise regulation of lipid metabolism,
316 vitamin and mineral metabolism, and molecular transport (Supplementary table 7).

317 **Discussion**

318 Lfs can be internalized by dermal fibroblasts, and SLs may influence effects
319 triggered by CbLf via changing the intracellular localization of internalized Lfs. SLs have
320 been shown to increase CbLf absorption by model skin (Ishii et al. 2012), probably via
321 facilitating transport of CbLf through multiple layers of the epidermis. However, SLs did
322 not affect the rate of CbLf internalization by HDFn as evident by internalization of ¹²⁵I-
323 labeled Lfs. Instead, SLs changed the intracellular localization of CbLf and may thus
324 affect its physiological functions. Compared with holo-forms of Lfs, all apo-forms of Lfs
325 displayed higher uptake, suggesting that conformation differences play a role in Lf
326 internalization by HDFn and may thereby modify effects of Lfs on dermal fibroblasts.
327 Apo-Lf has less compact conformation than holo-Lf (Baker and Baker 2004), so the LfR
328 binding site in apo-Lf may have higher chances to access the LfR on the membrane of
329 HDFn, leading to higher uptake of apo-Lf by HDFn.

CbLf and CbLf-SLs-assembly significantly up-regulated transcription of genes involved in cellular proliferation. Major proliferation-related signaling pathways activated by CbLf and CbLf-SLs-assembly were PI3K/AKT, platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF-1) signaling pathways. Additionally, the assembly activated VEGF (vascular endothelial growth factor) signaling. Lf has been reported to stimulate proliferation of dermal fibroblasts (Ishii et al. 2012), and induced proliferation of keratinocytes via activating the ERK signaling pathway (Tang et al. 2010). Fibroblasts are the predominant cells in the dermis, and they play essential roles in maintaining skin homeostasis and forming a heterogeneous cell population which produces ECM components including collagens, elastin, and glycoproteins (Martin 1997). A complex ECM network organized by dermal fibroblasts provides water retention and remarkable biomechanical properties to skin (Vedrenne et al. 2012). Renewal of the epidermis is much slower in elderly due to the unique extension of cell cycles (Kohl et al. 2011), and aging fibroblasts display a slower rate of proliferation than fetal fibroblasts (Khorramizadeh et al. 1999), suggesting that chronological aging at least partly results from decreased replicative capacity of dermal fibroblasts. Thus, promoted proliferation of dermal fibroblasts may result in improvement of skin health through increasing generation of ECM components and stimulating renewal of the epidermis.

CbLf and CbLf-SLs-assembly may be involved in facilitating migration of dermal fibroblasts (Supplementary table 1) via stimulating expression of ECM and related transcription factors, as well as growth factors. The migration of human skin cells is very complex, and it is crucial for skin wound healing and remodeling. Platelet derived growth factor (PDGF) is well characterized for its functions on growth and motility of

353 dermal fibroblasts, and it is believed to participate in wound healing processes (Li et al.
354 2004). CbLf and CbLf-SLs-assembly dramatically stimulated transcription of both PDGF
355 and PDGF receptor (PDGFRB), suggesting that they may be associated with
356 enhancement of cell migration via increasing PDGF transcription. In addition to PDGF,
357 CbLf and the assembly also enhanced transcription of FGF, another important growth
358 factor related to cell motility (Li et al. 2004). Two recombinant hLfs have been shown to
359 promote wound repair via stimulation of cell proliferation and migration of dermal
360 fibroblasts, as well as synthesis of ECM components (Engelmayer et al. 2008;
361 Takayama and Aoki 2012; Tang et al. 2010). Cell migration and proliferation are two
362 essential steps for re-epithelialization in skin wound healing and remodeling (Clark et al.
363 1996). CbLf and CbLf-SLs-assembly significantly enhanced transcription of genes
364 tightly related to cell migration and proliferation in dermal fibroblasts, implying that Lf
365 may play important roles in wound repair.

366 CbLf and CbLf-SLs-assembly remarkably promoted transcription of genes
367 involved in synthesis of extracellular matrix via enhancing transcription of ECM
368 components, promoting lysyl hydroxylation via stimulating transcription of related
369 enzymes, and inhibiting collagen degradation by increasing transcription of
370 metalloproteinase inhibitor 3 (Supplementary table 1). CbLf has been documented to
371 increase secretion of hyaluronan by increasing transcription of hyaluronan synthase 2
372 (HAS2), and elevate COL1A1 transcription in normal human dermal fibroblasts (Saito et
373 al. 2011). Consistently, transcription of HAS2 and collagen I was considerably up-
374 regulated by CbLf and CbLf-SLs-assembly. In particular, the effect of the assembly on
375 HAS2, a major hyaluronan synthase in dermis, was more potent than that of CbLf.

376 Collagens I and III are the most abundant collagens present in skin, and lysyl
377 hydroxylation is needed for cross-link formation to stabilize collagen networks in the
378 extracellular space (Krieg and Aumailley 2011), whereas hyaluronic acid fills the
379 connective tissue and is responsible for its strength (Delalle-Lozica 2010). Photo-aged
380 skin contains an abundance of degraded and disorganized collagen fibrils and has a
381 reduced production of type I and type III procollagen (Fisher et al. 2002; Talwar et al.
382 1995). Increased collagen synthesis or protection from collagen breakdown are
383 potential mechanisms for anti-aging effects (Chiu and Kimball 2003).

384 CbLf and CbLf-SLs-assembly may protect skin from oxidative stress via
385 stimulating expression of related genes and activating anti-oxidative signaling pathways.
386 Covering the entire outer surface of the body, human skin is the largest organ and is
387 constantly exposed to sunlight stress, including ultraviolet (UV) light irradiation. Reactive
388 oxygen species (ROS) damage cellular DNA, lipids, and proteins (Berlett and Stadtman
389 1997). There are two types of skin aging; one is chronological aging, which is due to the
390 passage of time, and another is premature aging or photo-aging owing to environmental
391 aggressors (Iddamalgoda et al. 2008). ROS generated from oxidative cell metabolism
392 and UV-exposure cause oxidative damage to cellular components, such as cell walls,
393 lipid membranes, mitochondria, and DNA. ROS facilitates both photo-aging and
394 chronological aging (Fisher et al. 2002). Based on the free radical theory, excess ROS
395 generated from oxidative metabolism result in cellular damage, which is a major cause
396 of aging (Hensley and Floyd 2002). CbLf and the assembly significantly activated the
397 NRF2-mediated oxidative stress response and antioxidant action of vitamin C to exert
398 their anti-oxidant effects. Nrf2 is a transcription factor known to initiate a cellular

399 response to defend cells against the deleterious effects of environmental toxicants
400 (Kensler et al. 2007). Vitamin C is an electron donor, and it eliminates most ROS
401 (Padayatty et al. 2003). CbLf and CbLf-SLs-assembly may remove ROS through
402 activating these two signaling pathways and thus diminish both photo-aging and
403 chronological aging to some extent. In agreement with our results, an *in vivo* study
404 conducted in hairless mice found that oral supplementation with Lf prevented UV-
405 induced skin damage (Murata et al. 2014). Consequently, CbLf and CbLf-SLs-assembly
406 treatments may lead to healthy and younger looking skin.

407 CbLf and CbLf-SLs-assembly may play roles in immunomodulatory activities by
408 enhancing transcription of chemokines, cytokines, and related enzymes. Skin is an
409 effective barrier between the organism and the environment to prevent invasion of
410 pathogens and to protect against toxins (Proksch et al. 2008). CbLf and CbLf-SLs-
411 assembly may activate IL-6, iNOS, Tweak, IL17A, and IL-8 signaling pathways to
412 prepare or protect dermal fibroblasts against microbial invasion (Supplementary table 3),
413 which may be beneficial for facilitating the wound healing process by reducing the risk
414 of infection. The antimicrobial activities and immunomodulatory effects of Lf have been
415 extensively documented (Siqueiros-Cendon et al. 2014; Vogel 2012; Wakabayashi et al.
416 2014). Microbiological contamination happens easily in cosmetics with high water
417 content, and it causes potential harmful effects to the consumers (Lundov et al. 2009).
418 Addition of Lf to cosmetics may not only prevent microbiological contamination of the
419 product, but also protect the skin from insults from bacteria and viruses.

420 CbLf, CbLf-SLs-assembly, and SLs may all upregulate cholesterol biosynthesis
421 of HDFn. The enhanced cholesterol synthesis may be associated with up-regulation of

hyaluronan synthesis as indicated in Supplementary table 1, since activities of hyaluronan synthase rely on lipid (Ontong et al. 2014). Moreover, cholesterol is an important ingredient of cell membrane, and it is essential to maintain permeability, fluidity, and protein functions of cell membrane (Espenshade and Hughes 2007). Thus, upregulation of cholesterol may be beneficial for the skin. Additionally, SLs were reported to reduce subcutaneous fat accumulation by increasing leptin synthesis in adipocytes (Lourith and Kanlayavattanakul 2009). Therefore, further studies using animal models are needed to clarify effects of SLs on lipid metabolism.

In addition to the effects on cholesterol biosynthesis, SLs alone were involved in synthesis of sex hormones and vitamins (Fig. 10.). Human skin is both a hormone target and an endocrine gland (Slominski et al. 2013). Estrogens shows a serial positive effects on human skin, such as protection against skin aging, and acceleration of wound healing (Thornton 2005). Moreover, SLs may stimulate synthesis of vitamin A and D, which also have favorable effects on skin. For examples, vitamin A and vitamin D both mediate proliferation and differentiation of skin epithelial cells (Bikle 2015; Randolph and Simon 1997). There are no reported effects of SLs on the synthesis of sex hormones and vitamins, and these effects need to be further validated.

Taken together, Lf is absorbed by dermal fibroblasts and may exert beneficial effects on skin health; SLs did not significantly influence internalization and bioactivities of Lf on dermal fibroblasts. Anti-oxidation effects, immunomodulatory activities, and enhancement of wound healing are major possible effects of Lf on dermal fibroblasts (Fig. 7). Lf may exert beneficial roles in skin health by stimulation of proliferation, promotion of life span of dermal fibroblasts, enhancement of synthesis of ECM

components, and increase in oxidative stress resistance. Moreover, Lf may stimulate wound repair as Lf up-regulated transcription of genes involved in promotion of proliferation, and migration of dermal fibroblasts, as well as enhancement of production of ECM components. Topical application of Lf to skin should be further investigated by evaluating changes at the protein level and also *in vivo* experiments, i.e. animal and human studies.

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Fig. 1.

Fig.1. Uptake of Lfs by HDFn. After D10 HDFn were incubated in supplement-free medium containing 125I-labeled Lf samples (100 µg/mL with or without SLs: 10 µg/mL) for 30 min at 37°C, cell-associated radioactivity was quantified in a gamma counter. Data are shown as means ± SD for three independent experiments (n=5). Differences between Lf and Lf-SLs-assembly groups were analyzed using one-way ANOVA. *p<0.001

254x190mm (96 x 96 DPI)

Fig. 2. Subcellular localization of internalized Lf in the presence or absence of SL. After cells were treated with Lfs (100 μ g/mL) or Lf-SLs-assemblies in supplement free medium for 30 min at 37°C, cells were stained with green fluorescence (Lf) and red fluorescence (nucleus). (A&B): hLf, (C&D): bLf, (E&F): CbLf. Data are shown as means $\pm$ SD for three independent experiments (n=3). Differences between Lf and Lf-SLs-assembly groups were analyzed using one-way ANOVA. Scale bar: 10 μ m. * p <0.001, # p <0.05, ** p <0.01.

254x190mm (96 x 96 DPI)

Fig. 3.

Fig. 3. Clustering of genes expressed in HDFs treated with Lf and SLs. Hierarchical clustering of 12 samples based on expression levels of 13716 probes detected in the microarray. Samples were clustered with a centroid linkage method on the horizontal axis, and the lengths of the branches were measure by a Euclidean algorithm. The vertical axis represents the degree of correlation between samples, and the lengths of the branches indicate similarities between samples or genes.

254x190mm (96 x 96 DPI)

Fig. 4. Heatmap representation of the genes significantly regulated by CbLf, CbLf-SLs-assembly, and SLs. Probe sets for genes significantly regulated (adjusted p value <0.05, fold change ≥ 3.0 or ≤ 0.3) were selected and reported in a heatmap. Each row represents a different probe set, and columns pertain to data collected from HDFn treated with CbLf, SLs, or CbLf-SLs-assembly.

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Fig. 5. Global analysis of transcriptomic changes in the presence of CbLf, SLs, and CbLf-SLs-assembly indicated by Venn diagrams. Venn diagrams represent genes significantly regulated by CbLf, SLs, and CbLf-SLs-assembly. Differentially expressed genes were selected following three criteria: fold change >1.5 for up-regulation, fold-change < 0.5 for down-regulation, and detection probability greater than 0.95 in all samples.

254x190mm (96 x 96 DPI)

Fig. 6.

Fig. 6. Real-time PCR verification of microarray results. (A) FABP3 (B) COL7A1 (C) CXCL6 (D) PLIN2 (E) CPA4 (F) EDN1. (G) Associations between results from microarrays and RT-PCR were analyzed, and all were shown to have $R^2 > 0.95$. Values are shown as mean fold-changes $\pm$ SD for three independent experiments ($n=5$), relative to control (non-treated control, set to 1). * $p < 0.001$, ** $p < 0.01$

254x190mm (96 x 96 DPI)

Fig. 7.

Fig. 7. Plausible physiological functions of topically applied CbLf and CbLf-SLs-assembly through activation of HDFn. According to the pathway analysis on microarray data, Lf is likely to activate HDFn to exert multiple functions such as anti-aging, wound healing, anti-microbial, cell proliferation, and cholesterol biosynthesis facilitating effects.

254x190mm (96 x 96 DPI)

Table 1 Primers for Real-time PCR

Primers	Sequence
FABBP3 Forward	AGCCTAGCCCAGCATCACTA
FABBP3 Reverse	AACCCACACCGAGTGACTTC
COL7A1 Forward	CCCATGAGGCACCAGATACT
COL7A1 Reverse	GCTGACTCCACCTTCGAGAC
CXCL6 Forward	TGTTTACGCGTTACGCTGAG
CXCL6 Reverse	AACTTGCTTCCCGTTCTTCA
PLIN2 Forward	ACTGGCTGGTAGGTCCCTTT
PLIN2 Reverse	GTCTCCTGGCTGCTCTTGTC
CPA4 Forward	TCGGAAGTGGAGGTGAAATC
CPA4 Reverse	GTAGCTGTGCAGGTCGATGA
EDN1 Forward	TCCTCTGCTGGTTCCTGACT
EDN1 Reverse	ATGGAAGCCAGTGAAGATGG
GAPDH Forward	CAACGAATTTGGCTACAGCA
GAPDH Reverse	GGTGGTCCAGGGGTCTTACT