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The mucin protein MUCL1 regulates melanogenesis and melanoma genes in a manner dependent on threonine content*

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The data that support the findings of this study are openly available and materials or data can be requested from the corresponding author.

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Summary

Background The regulation of melanogenesis has been investigated as a long-held aim for pharmaceutical manipulations with denotations for malignancy of melanoma. Mucins have a protective function in epithelial organs; however, in the most outer organ, the skin, the role of mucins has not been studied enough.

Objectives Our initial hypothesis developed from the identification of correlations between pigmentation and expressions of skin mucins, particularly those existing in skin tissue. We aimed to investigate the action of mucins in human melanocytic cells.

Materials and methods The expression of mucin proteins in human skin was investigated using microarray data from the Human Protein Atlas consortium (HPA) and the Genotype-Tissue Expression consortium (GTEx) database. Mucin expression was measured at RNA and protein levels in melanoma cells. The findings were further validated and confirmed by analysis of independent experiments.

Results We found that the several mucin proteins showed expression in human skin cells and among these, mucin-like protein 1 (MUCL1) showed the highest expression and also clear negative correlation with melanogenesis in epidermal melanocytes. We confirmed the correlations between melanogenesis and MUCL1 by revealing negative correlations in melanocytes with different melanin production, resulting from increased composition of threonine, mucin-conforming amino acid, and increased autophagy-related forkhead-box O signalling. Furthermore, threonine itself affects melanogenesis and metastatic activity in melanoma cells.

Conclusions We identified a significant association between MUCL1 and threonine with melanogenesis and metastasis-related genes in melanoma cells. Our results define a novel mechanism of mucin regulation, suggesting diagnostic and preventive roles of MUCL1 in cutaneous melanoma.

What is already known about this topic?

- Despite considerable advances in radioactive therapeutics or chemotherapeutic approaches for the treatment of abnormal melanogenesis, there are still many caveats to delivery, effectiveness and safety, thus leaving a necessity for more immediate pharmaceutical targets.
- Mucins have protective and chemical barrier functions in epithelial organs; however, in the skin, mucin has scarce expression and is known only as a diagnostic aid in skin disorders such as mucinosis.

What does this study add?

- We provide detailed analysis demonstrating the potential of mucin-like protein 1 (MUCL1), which showed negative correlations in melanocytes with different

melanin production, resulting from increased composition of threonine and increased autophagy-related forkhead-box O signalling in epidermal melanocytes and melanoma cells.
- We established that through an alternative pathway for MUCL1 biosynthesis, threonine supplementation recovers MUCL1 levels in melanoma. Changes, brought on by the essential amino acid threonine, resulted in substantial modulations in melanogenesis and reduced metastasis-related genes.

What is the translational message?

- This study demonstrates for the first time that the mucin protein of skin cells is compounded by distorted mucin homeostasis, with major effects on melanogenesis and metastasis-related genes in melanoma.
- We anticipate that these novel findings will be of keen interest to the community of scientists and medical practitioners examining skin dysfunction.

As the outermost organ of the human body, the skin is the first line of the defence system, acting as a physical immune barrier. During epidermal development, epithelial cells convert from a single layer into multiple layers and persistently migrate outward, and they are maintained and renewed throughout life.[1] Mucins are essential components that act as lubrication for cells, forming chemical barriers and binding to pathogens as part of the immune system.[2] There are two types of mucins, secreted and membrane-bound mucins. The secreted mucins form oligomeric structures and produce a thick viscous gel that lines and lubricates most luminal surfaces, and membrane-bound mucins are monomeric glycoproteins located primarily on the cell surface to act as a barrier against infection, and also inflammation.[3,4] As a cell-surface-associated mucin, MUC1 is expressed by skin fibroblasts acting in cell adhesion and migration.[5] Despite the important functions of mucins in defence against bacterial and external infections,[6,7] it has been reported that mucin proteins show restricted expression in normal skin.[8–10] On the other hand, some mucin genes have been detected that exhibit different expression patterns during human embryonic and fetal organ development than in adult tissues.[3] For example, MUC5AC is expressed in rat embryonic epidermis and may play a protective role in embryonic skin prior to birth before the mucus is replaced with hair.[11] MUC4 also exists in the skin of embryos and disappears late in gestation, which is consistent with a protective role for mucin in human embryos.[12] Based on these reports, we speculate that mucins appear in embryonic skin to play protective and defensive roles but that mucin expression in adult skin is restricted to reduce the risk of cancer progression.[13–15]

Abnormally increased melanogenesis and melanin accumulation within the epidermis is one of the strongest predictors of hyperpigmentation disorders and of high risk of the skin cancer melanoma.[16] Melanoma remains one of the most aggressive tumour types, mainly because of its propensity to metastasize and resist therapy. In this respect, it is important to alleviate excessive melanogenesis to attenuate the skin ageing-related phenotype and melanoma progression. Because melanin production and accumulation are connected with innate immunity and the external environment,[17] the skin barrier function may be a pivotal factor in melanogenesis regulation. However, the relationship of the mechanical state or barrier function of the skin with melanogenesis is poorly understood.

Mucin proteins have emerged as important molecules in sustaining health and in ameliorating or protecting against inflammation and cancer. Mucins have also begun to be used as specific diagnostic markers and therapeutic targets for numerous malignancies. However, the roles of mucin proteins in cutaneous physiological functions have remained mostly unclear. As mucin proteins have various regulatory roles in intracellular signalling events and adaptors, cellular survival, apoptosis, adhesion and metastasis, the positive effects of mucin in skin need to be investigated. In this study, we highlight the importance of skin mucins in neonatal human epidermal melanocyte (NHEM) and melanoma cells, MNT1 for melanogenesis and antitumour immunity.

Materials and methods

Antibodies and reagents

Antibodies against endomucin (V.7C7), MITF (C5), tyrosinase (H-109), TYRP1 (H-90), DCT (C-9) and GAPDH (FL-335) were obtained from Santa Cruz Biotechnology (Paso Robles, CA, USA). Antibodies against mucin-like protein 1 (MUCL1) (#2028680, MyBioSource, San Diego, CA, USA), MITF (Neomarkers, Fremont, CA, USA), tyrosinase (Upstate Biotechnology, Lake Placid, NY, USA) and EDNRB (#117529, Abcam, Waltham, MA, USA) were also used. MUC1 (#14161), Notch1 (#3608), p75NTR (#8238), FoxO3a (#2497), FoxO1 (#2880), SCF (#2093), SOX10 (#89356), SOD2 (#13141), GABARAP (#26632), LC3B (#43566) and ATG7 (#8558) antibodies were acquired from Cell Signaling Technology (Beverly, MA, USA). Antibodies were also used for precise detection of MUCL1 (NBP1-92366, Novus Biologicals, Littleton, CO, USA), MUCL1 (#364977, Usbio, Salem, MA,

USA), MUCL1 (#104968, Abbexa, Cambridge, UK) and MUCL1 (orb455304, Biorbyt, Cambridge, UK). Secondary antibodies for Western blot and immunofluorescence analyses were obtained from Cell Signaling Technology and Invitrogen (Carlsbad, CA, USA), respectively. Myc-DDK-MUCL1 (RC213754) and MUCL1 WT (SC305756) plasmids were obtained from OriGene (Rockville, MD, USA). A MUCL1 Validated Stealth RNAi DuoPak and scramble RNAi (#1299001) were acquired from Thermo Fisher Scientific (Waltham, MA, USA). Threonine-depleted cell culture medium was configured and prepared from Welgene (Gyeongsangbuk-do, Korea).

Cell culture and growth activity assay

Lightly, moderately and darkly pigmented NHEM were procured from Cascade Biologics (Portland, OR, USA), maintained and passaged in Medium 254 (#M254500) supplemented with Human Melanocyte Growth Supplement (Life Technologies, Carlsbad, CA, USA), 10% fetal bovine serum (FBS), 100 U mL^{-1} penicillin G and 100 μg mL^{-1} streptomycin sulfate. NHEM were incubated at 37°C with 5% CO_2, regularly passaged at a density of 80% (1 : 8 ratio), and we used NHEM with passage numbers 2–3 for experiments. MNT1 cells were maintained in Minimum Essential Medium (Gibco, New York, NY, USA) containing 10% Dulbecco's modified Eagle's medium, 20 mmol L^{-1} HEPES (Sigma-Aldrich, St Louis, MO, USA), 20% FBS, 100 U mL^{-1} penicillin G and 100 μg mL^{-1} streptomycin sulfate. The MNT1 cells were incubated at 37°C with 5% CO_2 and regularly passaged at a density of 80% (1 : 8 ratio). Cell proliferation and cytotoxicity were measured using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan).

Tyrosinase enzymatic activity assay and determination of melanin levels

To measure cellular tyrosinase activity, equal amounts of cell lysates (10 μg) were incubated with 10 mmol L^{-1} L-dihydroxyphenylalanine (L-DOPA) (pH 6·8) at 37°C for 1 h. Melanin synthesized from L-DOPA by tyrosinase in the cell extracts was measured at 490 nm in a microplate reader (Synergy H1, BioTek, Winooski, VT, USA). To measure cellular melanin levels, cell pellets were dissolved in 1 N sodium hydroxide, and the melanin levels were determined by measuring the absorbance at 490 nm. The melanin levels were normalized to the protein input.

Quantitative real-time reverse transcriptase polymerase chain reaction

Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. The RNA concentration was determined spectrophotometrically, and the integrity of the RNA was assessed using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Two micrograms of RNA were reverse-transcribed into cDNA using SuperScript III reverse transcriptase (RT) (Thermo Fisher Scientific) and aliquots were stored at −20°C. TaqMan RT-PCR (reverse transcriptase polymerase chain reaction) technology (7500Fast, Applied Biosystems, Foster City, CA, USA) was used to determine the expression levels of selected target genes with TaqMan site-specific primers and probes. The process included a denaturing step performed at 95°C for 10 min followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. The reactions were performed in triplicate. The mRNA expression levels were quantified using the relative C_T method and were normalized to the *GAPDH* levels.

Enzyme-linked immunosorbent assay

Medium was harvested and centrifuged for 15 min at 4°C. The supernatants were freeze-dried and used for mucin protein measurement with an enzyme-linked immunosorbent assay (ELISA) kit. The MUC1, endomucin and MUCL1 levels were quantified using MUC1 (#574100), endomucin (#534025) and MUCL1 (#153020) ELISA kits, respectively, according to the manufacturer's instructions (Abbexa).

Small interfering RNA and plasmid transfection

NHEM cultured in 60-mm dishes were transfected with Validated Stealth RNAi DuoPak MUCL1 small interfering RNA (siRNA) (#1299001) and a Stealth RNAi siRNA negative control (#12935100) using Lipofectamine RNAi MAX and 5 nmol L^{-1} siRNA for 48 h according to the manufacturer's instructions (Thermo Fisher Scientific). For forced expression of MUCL1, MNT1 cells ($3·5 \times 10^5$ cells per 6 wells) were grown to 60–70% confluence and then transfected with 4 μg of plasmid. The plasmids for expression of constitutively active Myc-DDK-MUCL1 (RC213754) and MUCL1 WT (SC305756) were obtained from OriGene.

Western blot analysis

To prepare cell lysates, cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in RIPA buffer [50 mmol L^{-1} Tris-HCl pH 7·4, 150 mmol L^{-1} NaCl, 0·5% sodium deoxycholate, 0·1% sodium dodecyl sulfate (SDS) and 1% NP-40] in the presence of a protease and phosphatase inhibitor cocktail (Sigma-Aldrich). The lysates were then centrifuged at 15 000 × **g** for 15 min, and the supernatants were used for analysis. The protein concentrations were determined using a BCA kit (Sigma-Aldrich) using bovine serum albumin as the standard. Equal amounts of protein (30 μg per well) from cell lysates were loaded, separated using 4–12% gradient SDS–polyacrylamide gel electrophoresis (PAGE), transferred onto polyvinylidene fluoride (PVDF) membranes and incubated with the appropriate antibodies. This incubation was followed by incubation with precleared protein G beads (GE Healthcare, Milwaukee, WI, USA) overnight at 4°C. Next, the beads were washed five times with lysis buffer. Western blotting was performed following standard protocols. The cell lysates were

boiled in SDS sample buffer and resolved using 4–12% SDS-PAGE. The proteins were then transferred to a PVDF membrane (Thermo Fisher Scientific) and probed using specific antibodies.

Assay for threonine levels in melanocytic cells

The concentration of threonine was measured according to manufacturer's protocol. Briefly, cells were washed with ice-cold PBS and lysed in RIPA buffer in the presence of a protease and phosphatase inhibitor cocktail (Sigma-Aldrich). The lysates were then rapidly homogenized on ice with 100 μL ice-cold assay buffer and centrifuged at 15 000 × *g* for 10 min at 4°C, and the supernatants were used for measurement. Threonine contents were measured with threonine assay kit (ab239726, Abcam) with normalization with total protein concentration (Pierce™ BCA assay; Thermo Fisher Scientific).

Immunofluorescence assay

NHEM were fixed for 30 min in 4% paraformaldehyde, washed again, and incubated for 10 min in 0·1% Triton X-100. The cells were washed three times in PBS and incubated with an anti-MUCL1 (1 : 200) antibody diluted in Hank's solution (0·44 mmol L^{-1} KH_2PO_4, 5·37 mmol L^{-1} KCl, 0·34 mmol L^{-1} Na_2HPO_4, 136·89 mmol L^{-1} NaCl and 5·55 mmol L^{-1} D-glucose) at 4°C overnight. Secondary antibodies (Alexa Fluor 555-conjugated goat antirabbit) were added, and the cells were incubated for 1 h at room temperature. After washing, the coverslips were mounted onto glass slides, and the cells were visualized using a confocal laser scanning microscope (LSM800, Carl Zeiss, Oberkochen, Germany). DAPI (4′,6-diamidino-2-phenylindole) was used to counterstain the cell nuclei. The acquired images were analysed using ZEN software (ZEN blue, Carl Zeiss).

Statistical analysis

The data are expressed as the mean ± SD. The normality of the data was analysed using the Shapiro–Wilk test, and results between different groups were compared using one-way ANOVA (followed by Dunnett's post hoc test) or Student's t-test. For RT-qPCR, the data are shown as the mean ± SD of at least three triplicate measurements. The P-values generated via two-tailed Student's t-tests were used to compare ΔC_T values between the control and treatment groups. All statistical tests were two-sided, with the level of significance established at $P < 0{\cdot}05$. R software (version 4·0.5) was used for statistical analyses.

Results

MUCL1 is highly expressed in skin and showed negative correlation with melanin deposition

Mucins have pivotal roles in the immune response and can regulate the functions of innate and acquired immune responses.[18,19] Human melanocytes interact with the endocrine, immune and inflammatory systems, and we hypothesized that the expression of any mucin proteins may be correlated with melanogenesis in skin cells. To investigate whether mucins are involved in melanocyte differentiation, we evaluated the expression levels of mucin proteins in lightly pigmented, moderately pigmented and darkly pigmented NHEM and in MNT1, human melanoma cells. Firstly, we validated that melanin contents and the activity of a main enzyme of melanogenesis, tyrosinase, significantly increased with pigment accumulation under the same incubation circumstances (Figure 1a–C). According to the skin tissues and organs analysed using RNA-seq by the Human Protein Atlas consortium (HPA) and Genotype-Tissue Expression consortium (GTEx), we selected mucin proteins that showed expression in human skin tissue. Specifically, EMCN, MUC1, MUC15, MUC18, MUC4 and MUCL1 showed expression as follows: protein-transcripts per million correspond to mRNA levels by HPA or GTEx: EMCN (13·5, 10·3); MUC1 (4·9, 11·4); MUC15 (52·0, 23·3); MUC18 (11·2, 47·8); MUC4 (0·0, 0·0); MUCL1 (33·8, 804·0) in human skin (Figure 1d, e). Among these, MUC1 and MUCL1 exhibited elevated relative abundance in melanocytes and, especially, MUCL1 represented the highest expression compared with any other skin mucins (Figure 1e). In view of these results, we examined the gene expression of the epithelial mucins MUC1 and MUC4; cell-surface associated mucin, MUC15; the cell-surface glycoprotein, MUC18; the mucin-like sialoglycoprotein, endomucin; and the endothelial mucin, MUCL1. Of the mucins, *muc1*, *emcn* and *mucl1* showed decreasing expression levels with increasing pigment production, suggesting a negative relationship of the expression of these genes with melanin levels (Figure 1f–h). On the other hand, *muc4*, *muc15* and *muc18* were highly expressed only in melanoma cells and their expression was not markedly correlated with melanin content (Figure 1i–k). MUC4 showed specific expression in melanoma cells and is known to be related to tumour progression,[15,20,21] MUC15 is abnormally upregulated in melanoma and virus infection[14,18,22] and MUC18 is also known as the melanoma cell adhesion molecule, MCAM.[23] Because normal skin exhibits rather restricted expression of mucins, we investigated the actual protein expression levels and found that MUCL1 protein levels were abundant in skin and more clearly related to the pigmentation degree than MUC1 or EMCN levels in melanocytic cells (Figure 1l–o and Figure S1; see Supporting Information).

MUCL1 is a pivotal factor for melanogenesis and the essential role of threonine for MUCL1 regulation

To confirm that MUCL1 is involved in the melanogenic pathway, we introduced siRNAs against MUCL1 into moderately pigmented NHEM and verified their effects. As shown, melanin levels and tyrosinase activity were significantly increased in MUCL1-directed siRNA-treated cells compared with control or mock (siRNA negative control)-treated cells (Figure 2a–d and Figure S1). Moreover, melanin levels and tyrosinase

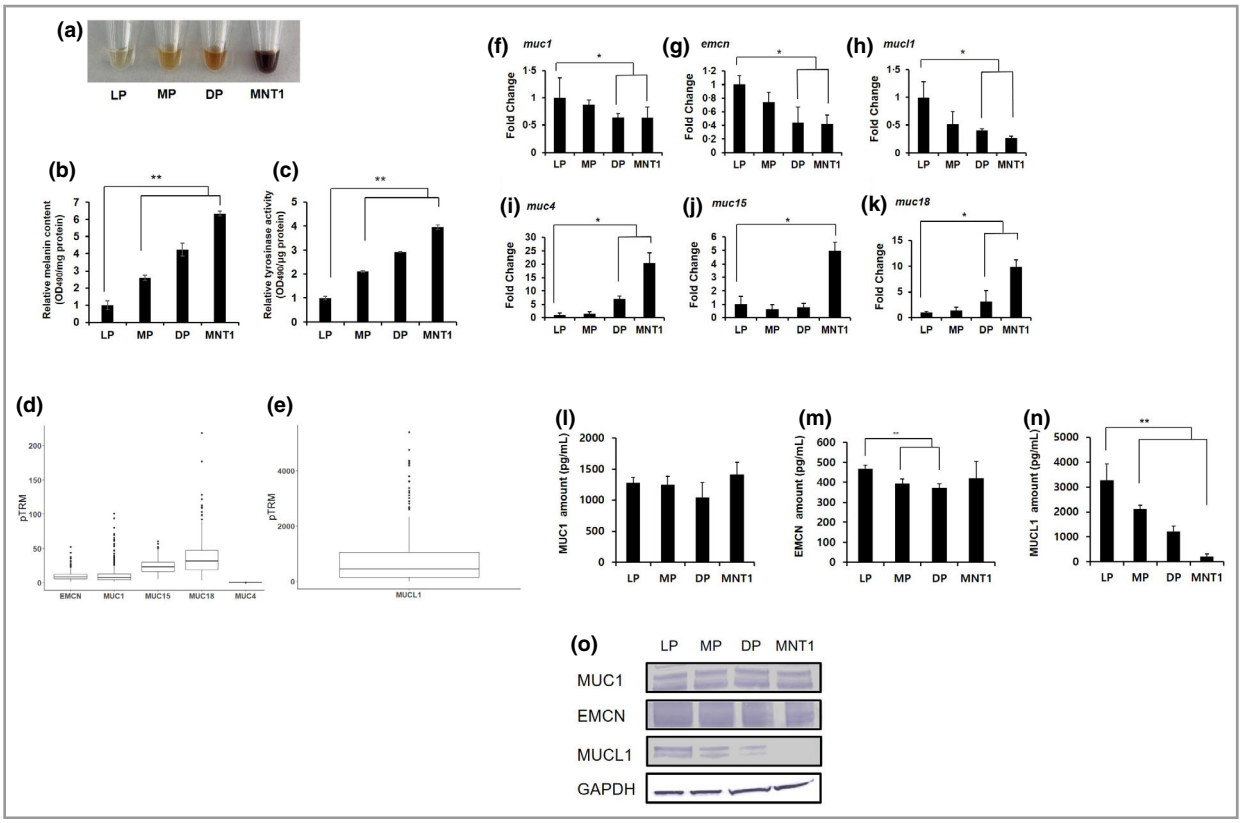

Figure 1 The expression of mucin proteins in pigmented cells and MUCL1 expression has a negative correlation with melanin production. (a) Melanin levels in lightly pigmented (LP), moderately pigmented (MP) and darkly pigmented (DP) melanocytes, and MNT1 melanoma cells were visualized after resuspension of cell pellets. (b) Melanin levels and (c) tyrosinase activity were assessed by measuring the absorbance at 490 nm with a spectrophotometer. (d) The expression levels of mucin proteins in skin tissue from the GTEx database ($N = 387$, lower leg). (e) The protein-transcripts level of MUCL1 by RNAseq was obtained from GTEx database ($N = 387$, lower leg). The mRNA expression levels of the mucin genes (f) *muc1*, (g) *emcn*, (h) *mucl1*, (i) *muc4*, (j) *muc15* and (k) *muc18* in LP, MP and DP melanocytes and MNT1 cells. The data represent three independent experiments by different melanocyte donors of NHEM or three replicates of MNT1 (mean ± SD). The expression levels of the mucin proteins (l) MUC1, (m) EMCN and (n) MUCL1. The data, produced in six independent experiments, are presented as the mean ± SD (pg mL^{-1}). (o) The protein expression levels of mucin proteins in pigmented cells were determined by Western blot analysis (see also Figure S1). *$P < 0{\cdot}01$ and **$P < 0{\cdot}001$ compared with the LP group.GTEx, Genotype-Tissue Expression consortium

activity were remarkably decreased in wild-type MUCL1-overexpressing (MUCL1 O/E) MNT1 cells (Figure 2e–h and Figure S1). These results suggest that MUCL1 affects melanogenesis meaningfully via its cellular expression.

Mature mucins are composed of two types of characteristic regions: the very lightly glycosylated amino- and carboxy-terminal regions and a large central region formed of multiple tandem repeats that account for up to half the content of the amino acids serine and threonine. To verify whether the amino acids in mucin composition could be responsible for the expression of MUCL1 in melanocytes and for the role on melanogenesis, we explored the influence of threonine or serine treatment under siMUCL1 (siRNAs against MUCL1) conditions. Interestingly, threonine (Figure 2i–k), but not serine (Figure 2l–n), reverted the increase of siMUCL1-induced melanogenesis. When threonine was added to siMUCL1-treated NHEM, melanin accumulation and tyrosinase activity were downregulated significantly. We found that threonine treatment reversed the decreased MUCL1 levels obtained by its silencing, without a significant effect of the threonine dose in cell viability (Figure 3a and Figure S2a; see Supporting Information), although serine supplementation did not alter MUCL1 expression (Figure 3b). When MUCL1-directed siRNAs were applied to cells, the expression of melanogenic genes, such as microphthalmia-associated transcription factor (MITF), tyrosinase (TYR), dopachrome tautomerase (DCT) and melanoma antigen recognized by T cells (MART1) were upregulated, whereas that of paired box transcription factor (PAX3), s100β, stem cell factor (SCF, and the SCF receptor cKIT) was downregulated (Figure 3c,d, Figure S2). We further examined the expression of several genes that have roles in early to late melanogenesis. NOTCH1, SRY-related HMG-box (SOX10), endothelin receptor B (EDNRB) and p75NTR expression was increased by siMUCL1 and threonine treatment significantly attenuated the elevations in the expression of these melanogenic genes (Figure 3e–h). Consistent with the modulation of melanogenesis-related genes, the protein levels of DCT, NOTCH1, EDNRB and P75NTR were also regulated by

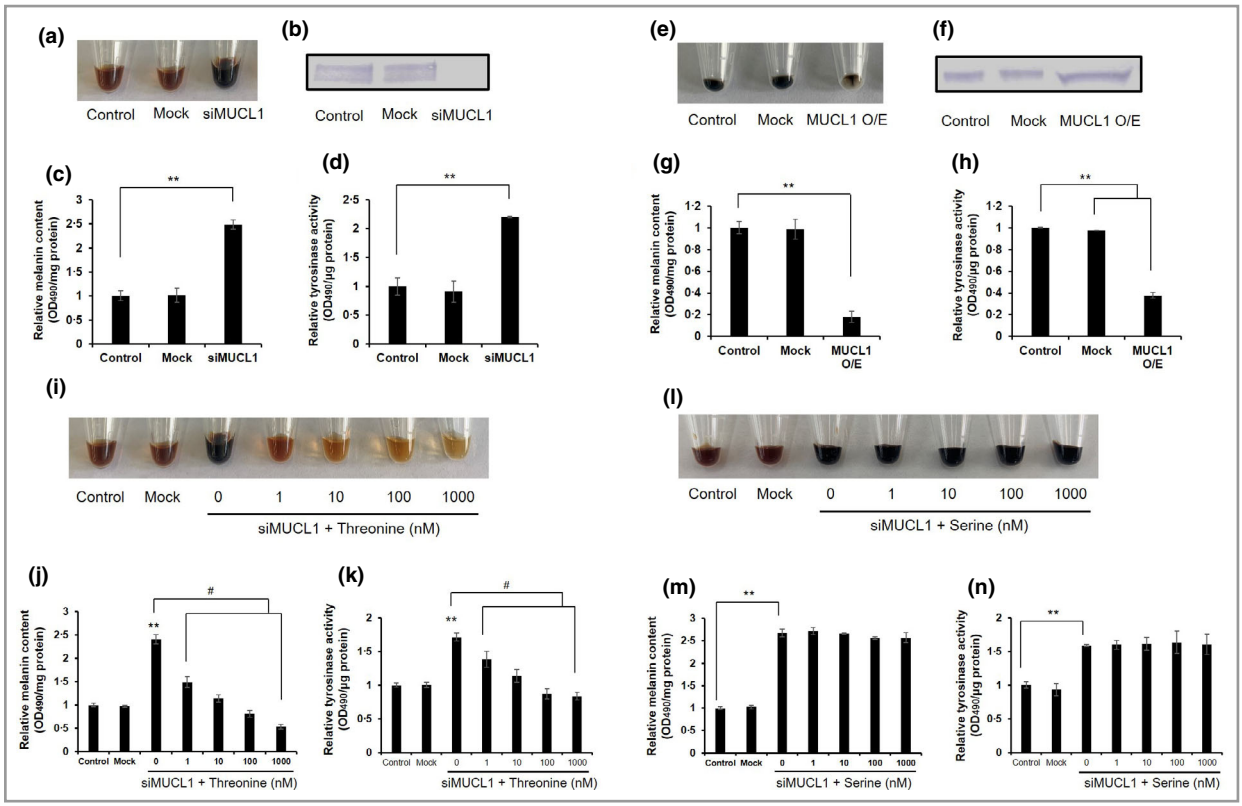

Figure 2 MUCL1 is a factor for melanogenesis regulation and is dependent on threonine content. The moderately pigmented (MP) neonatal human epidermal melanocytes (NHEM) were transfected with small interfering RNAs (siRNAs) against MUCL1 for 48 h. After treatment with the MUCL1 siRNAs, (a) melanin accumulation and (b) MUCL1 protein levels were visualized; and (c) melanin levels and (d) tyrosinase activity were measured. Control: untreated; mock: control siRNAs; siMUCL1: siRNAs against MUCL1. After overexpression of MUCL1, (e) melanin contents; (f) MUCL1 protein; (g) melanin levels; and (h) tyrosinase activity were visualized in MNT1 cells. Control: untreated; mock: empty vector-treated; MUCL1 O/E: overexpressing MUCL1 protein. The values are depicted as the mean ± SD from three independent experiments by different melanocyte donors of NHEM or three replicates of MNT1. $^{**}P < 0{\cdot}001$. MUCL1 siRNAs were applied, and the cells were then treated with 1–1000 nmol L^{-1} (i–j) threonine or (l–n) serine. (j, m) The melanin levels were visualized and quantified, and (K, N) tyrosinase activity was determined by measuring the absorbance at 490 nm. Control: vehicle-treated; mock: control siRNA-treated. The raw data for the Western blot are shown in Figure S1. The data shown are the mean ± SD from three independent experiments. $^{**}P < 0{\cdot}001$ compared with the control group; $^{\#}P < 0{\cdot}001$ compared with the siMUCL1 group.

siMUCL1 and threonine (Figure 3i and Figure S3; see Supporting Information), suggesting that MUCL1 negatively regulates melanogenesis and threonine treatment could ameliorate this abnormality by producing MUCL1 in NHEM. To confirm this, we measured the threonine level under siMUCL1-treated NHEM (Figure 3j) and also in wild-type MUCL1-overexpressing MNT1 cells (Figure 3k). As a result, the amount of MUCL1 did not affect the threonine amount, but only threonine regulates MUCL1 abundance. These results demonstrated that MUCL1 is a main regulator of melanogenesis and threonine affects pigmentation by increasing MUCL1 expression.

Threonine recovers hyperpigmentation by enhancing autophagy-related FoxO signalling under conditions of MUCL1 or threonine deprivation

As shown in the results using MUCL1-directed siRNA, threonine is associated with MUCL1-mediated melanogenesis; however, whether the level of threonine itself can modulate mucin protein expression and melanogenesis remains unclear. To evaluate the effects of threonine on melanogenesis, we applied threonine-depleted medium to moderately pigmented NHEM. When NHEM were cultured with threonine-depleted media, melanin generation and tyrosinase activity were largely increased and threonine treatment efficiently repaired this phenomena (Figure 4a–d and Figure S4a; see Supporting Information). Intriguingly, depletion of threonine reduced the levels of MUCL1 and EMCN, whereas the MUC1 level was elevated (Figure 4e–g). Under threonine-depleted conditions, the reduction in the protein expression of EMCN was ameliorated by threonine supplementation, and in the case of MUCL1, threonine increased the amount of protein more than the basal level (Figure 4e, f). Moreover, the mRNA expression levels of the melanogenic genes *mitf*, *tyr*, *dct*, *tyrp1*, *notch1*, *scf*, *sox10* and *pax3*, which were increased by threonine depletion, were downregulated by threonine application, whereas those of

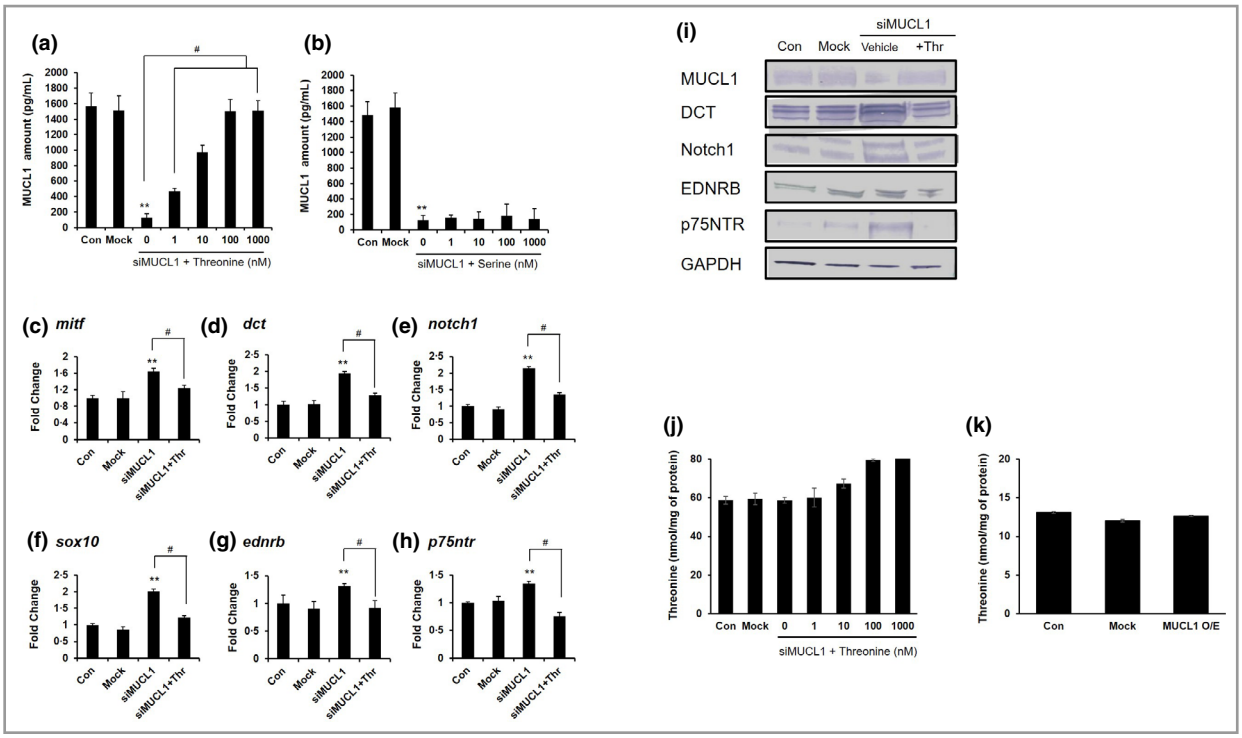

Figure 3 Threonine modulates MUCL1 and results in melanogenic gene expression. The amount of MUCL1 after treatment with (a) threonine or (b) serine under siMUCL1 (siRNAs against MUCL1) conditions. (c–h) Genes that were upregulated by treatment with siMUCL1 and addition of threonine were analysed by means of quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR). Control: vehicle-treated; mock: control siRNA-treated; siMUCL1: treated with siRNA against MUCL1; siMUCL1 + Thr: treated with siRNA against MUCL1 plus 100 nmol L^{-1} threonine. The data shown are the mean ± SD from three independent experiments by different melanocyte donors of NHEM or three replicates of MNT1. $^{**}P < 0{\cdot}001$ compared with the control group; $^{\#}P < 0{\cdot}001$ compared with the siMUCL1 group. (i) After treatment with MUCL1 siRNAs and threonine, the cell lysates were analysed by Western blot analysis. The raw data for the Western blot are shown in Figure S3. (j) The abundance of cellular threonine was measured as nmol per mg protein under siMUCL1 condition with threonine treatment in NHEM. (k) The amount of threonine after overexpression of MUCL1 in MNT1.

brn2, *s100β*, *ckit*, *mart1*, *ednrb* and *p75ntr* were not affected by threonine content (Figure 4h–o, Figure S4b). These results indicated that threonine itself modulates melanogenesis by the amount of tyrosinase activity and also expression of melanogenic genes. Furthermore, we investigated the cellular expression of MUCL1 by immunofluorescence assays and found that it was recovered after treatment with threonine to siMUCL1 status (Figure 5a).

The barrier function of mucin is closely related to mucosal immune homeostasis. Because autophagy plays a pivotal role in maintaining the integrity of the mucus barrier, defects in autophagy-related genes (ATG) increase the risk of inflammatory disease.[24–26] Because autophagy is involved in regulating the environment of skin cells and in the development of skin pigmentation diseases,[27,28] we assessed ATG expression and its intimate regulator FoxO signalling. FoxOs are vital modulators of cellular homeostasis processes, including regulation of autophagy, and FoxO3a acts as a melanogenesis regulator itself.[29,30] To uncover the action of threonine in autophagy–FoxO signalling, we estimated the gene expression of *foxo1*, *foxo3a*, the antioxidative genes *foxo3a* and *sod2*, and the ATG genes *gabarap*, *lc3b* and *atg7*. Mammalian GABARAPs and LC3 proteins are involved in canonical autophagy and play important roles in autophagy. GABARAPs facilitate membrane-bound factor transport and recruitment for autophagosomal maturation,[31] LC3 mobilizes cytosolic receptors,[32] and ATG7 is required for autophagosome formation.[33] The results of RT-qPCR and Western blotting showed that the gene and protein expression levels of FoxO1, FoxO3a, SOD2, and the ATG proteins GABARAP, LC3B and ATG7 were decreased in siMUCL1 or threonine-depleted conditions and threonine supplementation recovered that (Figure 5b–h and Figure S5; see Supporting Information).

Threonine attenuates excessive melanogenesis and metastasis-related gene expression in melanoma cells

FoxO transcription factors have been studied as tumour suppressors due to their capacity to modulate genes pivotal for cell migration and metastasis in various organs[34–37] and in uveal melanoma cancer cells.[38] Because threonine treatment diminished melanogenesis and activated FoxO-mediated autophagy signalling under conditions of mucin or also threonine deficiency, we tried to estimate the attenuation effects

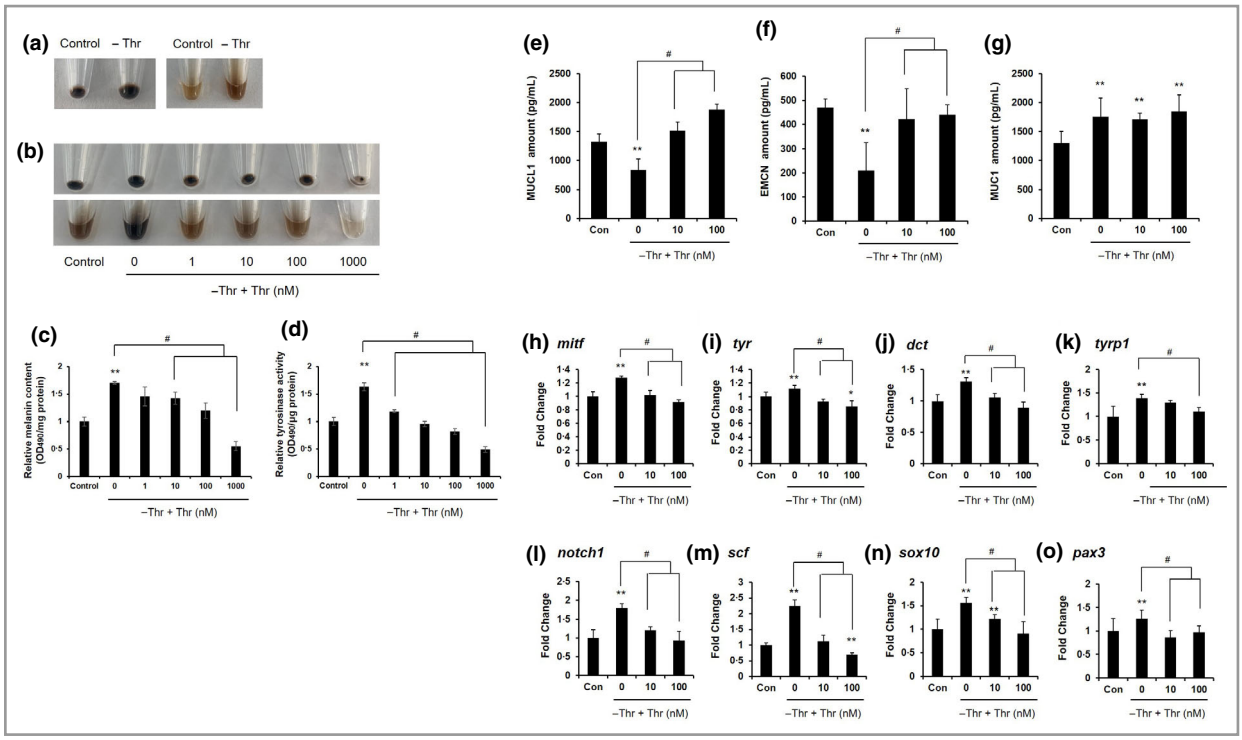

Figure 4 Threonine affects melanin formation via modulating cellular mucins and melanogenesis. (a) NHEM were treated with threonine-depleted medium and melanin was visualized as cell pellets and resuspension of cell pellets. (b) The melanin levels were visualized with supplementation of threonine under threonine-depleted conditions. (c) Melanin levels and (d) tyrosinase activity was quantified. (e–g) The expression levels of the (e) MUCL1, (f) EMCN and (g) MUC1 were measured. The data were produced from six independent experiments and are presented as the mean ± SD (pg mL^{-1}). (h–o) Melanogenic genes whose expression was altered by threonine depletion and the addition of threonine were analysed by means of quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR). Control: untreated; -Thr: treated with threonine-depleted medium. The data shown are the mean ± SD from three independent experiments by different melanocyte donors. $^{**}P < 0{\cdot}001$ compared with the control group; $^{\#}P < 0{\cdot}001$ compared with the threonine-depleted group.NHEM, neonatal human epidermal melanocytes

of melanoma malignancy. Threonine reduced melanin accumulation and tyrosinase activity effectively at the dose range of 1–1000 nmol L^{-1} (Figure 6a–c). As a result, threonine treatment decreased melanin production and tyrosinase activity to a similar extent under siMUCL1 and threonine-depleted conditions (Figures 2i–k and 4a–d). These results were supported by the expression levels of the representative melanogenic genes *mitf*, *tyr*, *dct*, *tyrp1*, *notch1* and *sox10*, which were reduced following the addition of threonine (Figure 6d–i). Surprisingly, among the melanogenic genes, *ckit* and its substrate *scf* exhibited increased expression levels with threonine application (Figure 6j, k). Previous studies have indicated that cKIT plays an active role in the progression of human melanoma and inhibits the malignancy of melanoma cells and that its substrate SCF increases cKIT expression, which results in melanoma cell apoptosis.[39–41] Threonine treatment might attenuate melanoma malignancy by reducing melanogenic genes and simultaneously inducing apoptosis of melanoma cells. Threonine increased the expression of the mucin genes *emcn* and *mucl1* but not *muc1*, which showed suppressed expression in MNT1 cells (Figure 6l–n). Moreover, in terms of the expression of mucin genes, which are abnormally upregulated in melanoma cells, *muc15* was not affected, and *muc4* and *muc18* presented decreased expression on threonine treatment (Figure 6o–q). MUC18 is the well-known cell adhesion molecule MCAM and MUC4 also engages in metastasis in a melanoma-specific manner.[42] Consistent with these findings, the amounts of the mucin protein EMCN and especially MUCL1 were dramatically increased by threonine treatment (Figure 6r–t). Our data suggest that the addition of threonine attenuates melanogenesis and the expression of some metastasis-related genes in melanoma cells and the melanoma suppression ability of threonine may contribute to enhancing mucin expression in mucin-deficient human melanoma cells.

Discussion

Previous studies have provided clues regarding melanogenesis regulation by specific amino acids and have revealed that supplementation with glutathione or cysteine provokes intermediate production, which imparts a range of yellowish to reddish colours to the skin.[43,44] The addition of cysteine or glutathione to DOPA quinone leads to the intermediate formation, followed by subsequent transformations and polymerization to the final product, pheomelanin.[45,46] In the absence of thiol compounds, DOPA quinone undergoes an

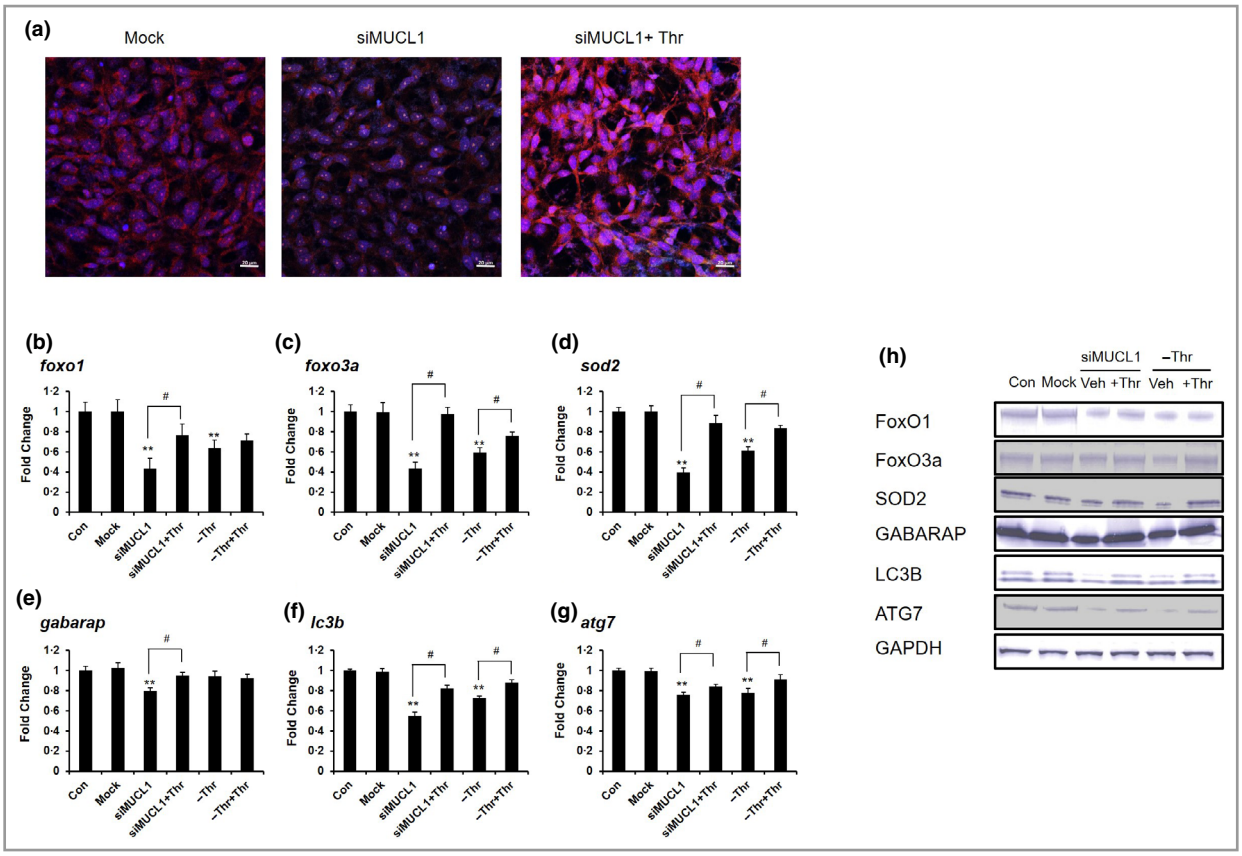

Figure 5 The addition of threonine restores the expression of MUCL1 and activates autophagy-related FoxO signalling. (a) NHEM were transfected with small interfering RNAs (siRNAs) against MUCL1, and 100 nmol L^{-1} threonine was added. The cells were stained with an anti-MUCL1 antibody (MUCL1: red), and 4′,6-diamidino-2-phenylindole (DAPI) was used to counterstain the cell nuclei (blue). Representative images were obtained under a confocal laser scanning microscope and merged. Mock, control siRNAs; siMUCL1, siRNAs against MUCL1; siMUCL1 + Thr, siRNAs against MUCL1 plus 100 nmol L^{-1} threonine. Scale bars, 20 μm. (b–g) Genes whose expression was downregulated by siMUCL1 or threonine depletion and elevated by threonine treatment were assessed. Control, vehicle-treated; mock, control siRNA-treated; siMUCL1, treated with siRNA against MUCL1; siMUCL1 + Thr, treated with siRNAs against MUCL1 plus 100 nmol L^{-1} threonine; –Thr, treated with threonine-depleted medium; –Thr + Thr, subjected to threonine depletion and 100 nmol L^{-1} threonine treatment. The data shown are the mean ± SD from three independent experiments by different melanocyte donors. $^{**}P < 0{\cdot}001$ compared with the control group; $^{\#}P < 0{\cdot}001$ compared with the siMUCL1 or threonine-depleted group. (h) After treatment with MUCL1 siRNAs or threonine depletion plus threonine treatment, the cell lysates were subjected to Western blot analysis. The raw data for the Western blot are shown in Figure S5.NHEM, neonatal human epidermal melanocytes

intramolecular cyclization and oxidation to form DOPA chrome, which is then converted to 5,6-dihydroxyindole (DHI) or 5,6-dihydroxyindole-2-carboxylic acid (DHICA). Eumelanin is formed by polymerization of DHI and DHICA and their quinones.[47] Because mucin production is impaired by the limited availability of certain amino acids under inflammatory conditions, threonine supply can accelerate mucin synthesis in animal models and predictions based on protein features.[48–50] Based on these reports, we hypothesized that when the mucin layer is disrupted from its normal condition, replenishment of threonine is a useful intervention for mucin restoration in skin melanocytic cells. Moreover, threonine itself impacted melanin and mucin protein construction, indicating that threonine supplementation provoked mucin expression. In human melanoma cells, certain amino acids accelerate proliferation, migration, aggressiveness and survival.[51] This may be because specific amino acids induce metabolic alterations to apoptosis without notably affecting other normal cells.[52] Based on previous reports, certain types of amino acids, small peptides and glycinamide show pigment-lightening effects in NHEM and melanoma cells,[53,54] and the biosynthetic pathways of various amino acids could be valuable therapeutic targets for changing protein generation in melanoma cells.[55] In addition, heterotopic expression of MUC18 in primary skin melanomas elevated their tumorigenicity and metastatic capacity *in vivo*.[56] Together with MUC18, MUC4 also participates in metastasis by exhibiting tumour progression-promoting activity in a melanoma-specific manner.

Recently, many studies have provided evidence that autophagy plays diverse roles in melanocyte biology.[57] In melanocytes, autophagy activation regulates melanogenesis and melanosome biogenesis, and autophagy induction modulates melanosome degradation.[58] Skin mucosal autophagy is

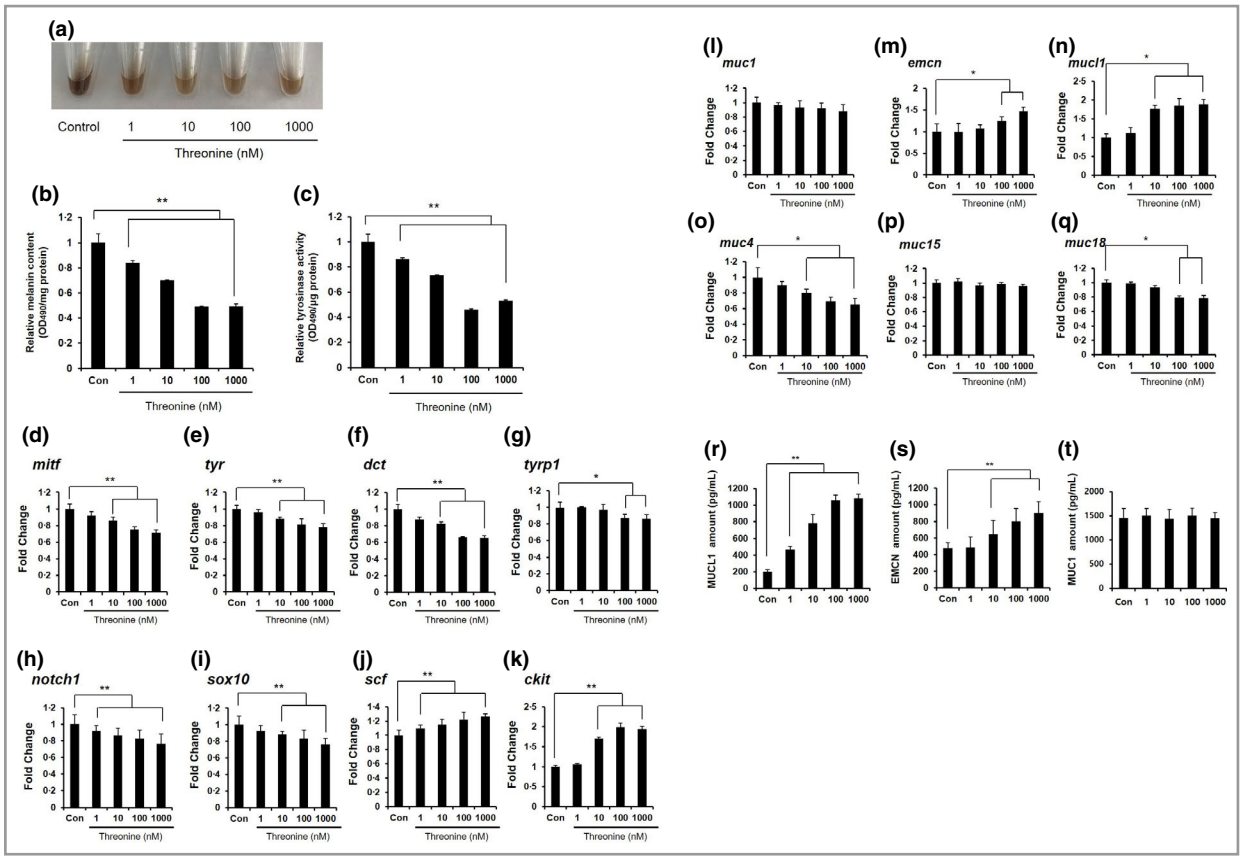

Figure 6 Threonine attenuates melanogenesis and metastasis-related gene expression. (a) The human melanoma cell MNT1 was treated with 1–1000 nmol L^{-1} threonine and melanin levels were visualized. (b) The melanin levels were assessed, and (c) tyrosinase activity was calculated compared with that in the control group. (d–k) The relative expression of melanogenic genes that were altered by threonine treatment is depicted. (l–q) The expression changes in mucin genes are indicated. The data shown are the mean ± SD from three independent experiments. (r–t) The expression levels of the mucin proteins MUCL1, EMCN and MUC1. The data, produced in six independent experiments, are presented as the mean ± SD (pg mL^{-1}). *$P < 0{\cdot}01$ and **$P < 0{\cdot}001$ compared with the control group.

controlled by autophagy-related factors such as gamma-aminobutyric acid receptor-associated protein (GABARAP), microtubule-associated protein 1 light chain 3B (LC3B) and autophagy-related protein 7 (Atg7) in the FoxO signalling pathway.[59] GABARAPs have essential roles in promoting autophagosomal maturation-related factor recruitment and transport, while LC3B plays an essential role in the recruitment of cytosolic receptors. Atg7 is needed for autophagosome formation and regulates ultraviolet radiation-induced inflammation and skin tumorigenesis. In addition, sustained redox states are critical to essential physiological processes, such as cell proliferation and the immune response. SOD2, also known as MnSOD, is a key antioxidative enzyme involved in cellular oxidative stress regulation and transcription of FoxO signalling pathway members. In this study, SOD2 expression was greatly reduced in NHEM following MUCL1 or threonine depletion and recovered by threonine supplementation. Functional impairment of autophagy results in elevated oxidative stress in cells, but antioxidants can elicit protective effects by increasing autophagic activity.[60] In this study, the gene and protein expression levels of FoxO1, FoxO3a and SOD2 were greatly decreased and the autophagy-related molecules GABARAP, LC3B and Atg7 were found to be reduced under insufficient conditions of MUCL1 or threonine. It is surprising that threonine could have recovered these gene expressions, especially *foxo3a*, *sod2* and *lc3b*, to the almost steady level and result in ascending autophagic activity against deficient status of mucin or threonine. Concomitant elevations in the levels of autophagic and antioxidative enzymes were observed, indicating that threonine intervention has a potential that activated FoxO-mediated autophagy to increase melanosome degradation and oxidative stimulation resistance in a mucin-deficient situation of skin, such as desiccation.

The mucin layer can elicit positive effects, such as protective effects on the skin barrier, except in cases of abnormal accumulation of mucins in specific regions. Mucins have broad pharmacological applications in drug delivery, and dysfunction of mucin synthesis can result in tumorigenesis and chronic inflammation.[61] Treatment with retinoic acid, which is well known as an efficient reagent for the treatment of skin senescence and photoageing, has been found to increase mucin deposition throughout the epidermis and dermis in a biopsy

study.[62] In addition to its protective functions derived from its physical and chemical characteristics, the mucin layer also has dynamic defence barrier functions. We expect that mucins interact closely with the skin-associated microbiota and serve as key regulators of skin homeostasis. Mucins are primarily composed of carbohydrate units that are conjugated to threonine and serine side chains. In the skin, mucins protrude into the adventitial dermis, and the mucin layer thickness is determined by the composition, synthesis and secretion processes. As shown in the results, threonine induced expression of the mucin proteins MUCL1 and EMCN, similar to the findings of a previous study with specific amino acids.[43] Intensification of the mucin layer upon threonine treatment might have contributed to the antimelanogenic effects in melanoma cells.

In conclusion, we provide evidence of MUCL1 actions in skin cells and consider that preventing melanogenesis via intervention with threonine is a meaningful target for hyperpigmentation and melanoma because of its safety and the convenience of supplementation.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1 Analyses of mucin protein expression in pigmented cells.

Figure S2 The effects of threonine treatment on cell viability and melanogenic gene expression under siMUCL1 conditions in NHEM.

Figure S3 The expression of melanogenic genes with MUCL1 siRNAs.

Figure S4 The effects of threonine treatment on cell viability and melanogenic gene expression under threonine-depleted conditions in NHEM.

Figure S5 Threonine recovers the decreased autophagy-related FoxO signalling on MUCL1 or threonine deprivation.