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

J. Kim and H. Choi

Amorepacific R&D Center, 1920 Yonggu-daero, Giheung-gu, Yongin-si, Gyeonggi-do, 17074, Korea

Linked Comment: C. Casalou and D.J. Tobin. Br J Dermatol 2022; 186:388–389.

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
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. Mucins are essential components that act as lubrication for cells, forming chemical barriers and binding to pathogens as part of the immune system. 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. A cell-surface-associated mucin, MUC1 is expressed by skin fibroblasts acting in cell adhesion and migration. Despite the important functions of mucins in defence against bacterial and external infections, it has been reported that mucin proteins show restricted expression in normal skin. 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. 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. 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. Based on these reports, we speculate that mucins appear in embryonic skin to play protective and defensive roles but that mucin expression restricted expression in normal skin. In adult skin is restricted to reduce the risk of cancer progression. 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. 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, 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. 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 (CS), 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) (#20631, 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 (NBPI-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 Biologies (Portland, OR, USA), maintained and passed 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% CO₂, regularly passed 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% CO₂ and regularly passed 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 1,2-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 × 10⁵ 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 x g for 10 min at 4°C, and the supernatants were used for measurement. Threonine contents were measured with threonine assay kit (ab339726, 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 KH₂PO₄, 5.37 mmol L⁻¹ KCl, 0.34 mmol L⁻¹ Na₂HPO₄, 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 ΔC₅₇ values between the control and treatment groups. All statistical tests were two-sided, with the level of significance established at P < 0.05. 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. 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. MUC15 is abnormally upregulated in melanoma and virus infection and MUC18 is also known as the melanoma cell adhesion molecule, MCAM. 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 as a regulator of 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
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
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, act, iyp1, notch1, scf, sox10 and pax3, which were increased by threonine depletion, were downregulated by threonine application, whereas those of
brn2, s100b, dct, mert1, 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,22,23 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 gabraps, 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).

FoxO transcription factors have been studied as tumour suppressors due to their capacity to modulate genes pivotal for cell migration and metastasis in various organs34–37 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
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 4](image-url)
intramolecular cyclization and oxidation to form DOPAchrome, 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. 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. 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. This may be because specific amino acids induce metabolic alterations to apoptosis without notably affecting other normal cells. Based on previous reports, certain types of amino acids, small peptides and glycinamide show pigment-lightening effects in NHEM and melanoma cells, and the biosynthetic pathways of various amino acids could be valuable therapeutic targets for changing protein generation in melanoma cells. In addition, heterotopic expression of MUC18 in primary skin melanomas elevated their tumorigenicity and metastatic capacity in vivo. 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. In melanocytes, autophagy activation regulates melanogenesis and melanosome biogenesis, and autophagy induction modulates melanosome degradation. Skin mucosal autophagy is...
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. 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 decreased 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. 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. 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...
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 MUC1 and EMCN, similar to the findings of a previous study with specific amino acids.\textsuperscript{43} Intensification of mucin proteins MUCL1 and EMCN, similar to the findings of a previous study with specific amino acids.\textsuperscript{43} The mucin layer upon threonine treatment might have contributed to the antimelanogenic effects in melanoma cells.

In conclusion, we provide evidence of MUC1 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.

Acknowledgments

We thank Dr Dong-hwa Choi in Biocenter, Gyeonggido Business & Science Accelerator for technical assistance with confocal microscopy.

References

1 Dekoninck S, Hannezo E, Sifrim A et al. Defining the design principles of skin epidermis postnatal growth. Cell 2020; \textit{181}:604–20.e22.
2 Marin F, Corstjens P, de Gaullejac B et al. Mucins and molluscan calcification. Molecular characterization of mucoprotein, a novel mucin-like protein from the nacreous shell layer of the fan mussel \textit{Pincta nobilis} (Bivalvia, pteriomorphia). J Biol Chem 2009; \textit{275}:20667–75.
3 Morrison CB, Markovetz MR, Ehre C. Mucus, mucins, and cystic fibrosis. Pediatr Pulmonol 2019; \textit{54} (Suppl. 3):S84–96.
4 Dhar P, McAuley J. The role of the cell surface mucin MUC1 as a barrier to infection and regulator of inflammation. Front Cell Infect Microbiol 2019; \textit{9}:117.
5 Kumar P, Ji J, Thirkill TL et al. MUC1 is expressed by human skin fibroblasts and plays a role in cell adhesion and migration. Biosearch Open Access 2014; 3:45–52.
6 Frenkel ES, Ribbeck K. Salivary mucins in host defense and disease prevention. J Oral Med Microbiol 2015; \textit{7}:29759.
7 Frenkel ES, Ribbeck K. Salivary mucins protect surfaces from colonization by cariogenic bacteria. Appl Environ Microbiol 2015; \textit{81}:332–8.
8 Boskey AL. Bio-mineralization: an overview. Connect Tissue Res 2003; \textit{44} (Suppl. 1):5–9.
9 Marin F, Luquet G, Marie B et al. Molluscan shell proteins: primary structure, origin, and evolution. Cur Top Dev Biol 2008; \textit{80}:209–76.
10 Kasprzak A, Adamek A. Mucins: the old, the new and the promising factors in hepatobiliary carcinogenesis. Int J Mol Sci 2019; \textit{20}:1288. https://doi.org/10.3390/ijms20061288.
11 Ferretti V, Segal-Irías A, Barbeito CG et al. Muc5ac mucin expression during rat skin development. Eur J Histocem 2015; \textit{59}:2462.
12 Zhang J, Yasin M, Carraway CA et al. MUC4 expression and localization in gastrointestinal tract and skin of human embryos. Tissue Cell 2006; \textit{38}:271–5.
13 Chakraborty S, Swanson BJ, Bonthu N et al. Aberrant upregulation of MUC4 mucin expression in cutaneous condyloma acuminatum and squamous cell carcinoma suggests a potential role in the diagnosis and therapy of skin diseases. J Clin Pathol 2010; \textit{63}:579–84.
14 Dai W, Liu J, Liu B et al. Systematical analysis of the cancer genome atlas database reveals EMCN/MUC15 combination as a prognostic signature for gastric cancer. Front Mol Biol 2020; \textit{7}:19.
15 Jonckheere N, Van Seuningen I. Integrative analysis of the Cancer Genome Atlas and Cancer Cell Lines Encyclopedia large-scale genomic databases: MUC4/MUC16/MUC20 signature is associated with poor survival in human carcinomas. J Transl Med 2018; \textit{16}:259.
16 Mujahid N, Liang Y, Murakami R et al. A UV-independent topical small-molecule approach for melanin production in human skin. Cell Rep 2017; \textit{19}:2177–84.
17 Koike S, Yamasaki K. Melanogenesis connection with innate immunity and toll-like receptors. Int J Mol Sci 2020; \textit{21}:9769. https://doi.org/10.3390/ijms21249769.
18 Chen ZG, Wang ZN, Yan Y et al. Upregulation of cell-surface mucin MUC15 in human nasal epithelial cells upon influenza A virus infection. BMC Infect Dis 2019; \textit{19}:622.
19 Li Y, Elmén I, Segota I et al. Prebiotic-induced anti-tumor immunity attenuates tumor growth. Cell Rep 2020; \textit{30}:1753–66.e6.
20 Kakkanessery V, Winterhalter S, Nick AC et al. Vascular-associated Muc4/Vwfl co-localization in human conjunctival malignant melanoma specimens-tumor metastasis by migration? Curr Eye Res 2017; \textit{42}:1382–8.
21 Workman HC, Sweeney C, Carraway KL 3rd. The membrane mucin Muc4 inhibits apoptosis induced by multiple insults via ErbB2-dependent and ErbB2-independent mechanisms. Cancer Res 2009; \textit{69}:2845–52.
22 Riker AI, Enkemann SA, Fodstad O et al. The gene expression profiles of primary and metastatic melanoma yields a transition point of tumor progression and metastasis. BMC Genom 2008; \textit{1}:13.
23 Feng R, Wang Y, Ramachandran V et al. Characterization of novel neutralizing mouse monoclonal antibody JM1-24-3 developed against MUC18 in metastatic melanoma. J Exp Clin Cancer Res 2020; \textit{39}:273.
24 Gu Y, Han J, Jiang C et al. Biomarkers, oxidative stress and autophagy in skin aging. Ageing Res Rev 2020; \textit{59}:101036.
25 Guo Y, Zhang X, Wu T et al. Autophagy in skin diseases. Dermatol 2019; \textit{215}:380–9. https://doi.org/10.1159/000500470.
26 Wang M, Charareh P, Lei X, Zhong JL. Autophagy: multiple mechanisms to protect skin from ultraviolet radiation-driven photoaging. Oxid Med Cell Longev 2019; \textit{2019}:8135985. https://doi.org/10.1155/2019/8135985.
27 Zhu W, Zhao Z, Cheng B. The role of autophagy in skin pigmentation. Eur J Dermatol 2020; \textit{30}:655–62.
28 Kim JY, Kim J, Ahn Y et al. Autophagy induction can regulate skin pigmentation by causing melanosome degradation in keratinocytes and melanocytes. Pigment Cell Mdmn Res 2020; \textit{33}:403–15.
29 Cheng Z. The FoxO-autophagy axis in health and disease. Trends Endocrinol Metab 2019; \textit{30}:658–71.
30 Kim J, Choi H, Cho EG et al. FoxO3a is an antimelanogenic factor that mediates antioxidant-induced depigmentation. J Invest Dermatol 2015; \textit{134}:1378–88.
31 Agrotis A, Pengo N, Burden JJ et al. Redundancy of human ATG4 protease isoforms in autophagy and LC3/GABARAP processing revealed in cells. Autophagy 2019; \textit{15}:976–97.
32 Schaaf MB, Keulers TG, Voojs MA et al. LC3/GABARAP family proteins: autophagy-(un)related functions. FASEB J 2016; \textit{30}:3961–78.
MUCL1 as a regulator of melanogenesis, J. Kim and H. Choi 543

33 Trentesaux C, Fraudeau M, Pitasi CL et al. Essential role for autophagy protein ATG7 in the maintenance of intestinal stem cell integrity. Proc Natl Acad Sci U S A 2020; 117:11336–46.

34 Jiramongkol Y, Iam EW. FOXO transcription factor family in cancer and metastasis. Cancer Metastasis Rev 2020; 39:681–709.

35 Hayes JD, Dinkova-Kostova AT, Tew KD. Oxidative stress in cancer. Curr Opin Hematol 2020; 28:167–97.

36 Park MK, Yao Y, Xia W et al. PTEN self-regulates through USP11 via the PI3K–FOXO pathway to stabilize tumor suppression. Nature Comm 2019; 10:636.

37 Coomans de Brachène A, Demoulin JB. FOXO transcription factors in cancer development and therapy. Cell Mol Life Sci 2016; 73:1159–72.

38 Yan F, Liao R, Farhan M et al. Elucidating the role of the FoxO3a transcription factor in the IGF-1-induced migration and invasion of uveal melanoma cancer cells. Biomed Pharmacother 2016; 84:1538–50.

39 Stankov K, Popovic S, Mikov M. C-KIT signaling in cancer treatment. Curr Pharmaceut Des 2014; 20:2849–80.

40 Luca MR, Bar-Eli M. Molecular changes in human melanoma metastasis. Histol Histopathol 1998; 13:1225–31.

41 Hue J, Kim A, Song H et al. IL-18 enhances SCF production of melanoma cells by regulating ROI and p38 MAPK activity. Immunol Lett 2005; 96:211–17.

42 Yu J, Xu L, Yan J et al. MUC4 isoforms expression profiling and prognosis value in Chinese melanoma patients. Clin Exp Med 2020; 20:299–311.

43 Faure M, Mettraux C, Moennoz D et al. Specific amino acids increase mucin synthesis and microtub aspirations in dextran sulfate sodium-treated rats. J Nutrition 2002; 119:1388–93.

44 Manning PL, Edwards NP, Bergmann U et al. Autophagy gene ATG7 regulates Pheomelanin pigment remnants mapped in fossils of an extinct mammal. Nat Comm 2019; 10:2250.

45 Napolitano A, Panzella L, Monfregola G et al. Pheomelanin-induced oxidative stress: bright and dark chemistry bridging red hair phenotype and melanoma. Pigment Cell Melanoma Res 2014; 27:721–33.

46 Agrup G, Hansson C, Rorsman H et al. The effect of cysteine on oxidation of tyrosine, DOPA, and cysteinyldopas. Arch Dermatol Res 1982; 272:103–15.

47 Mojamdar M, Ichihashi M, Mishima Y. Effect of DOPA-loading on glutathione-dependent S-S-cysteinyldopa genesis in melanoma cells in vitro. J Invest Dermatol 1982; 78:224–6.

48 Hearing VJ. Determination of melanin synthetic pathways. J Invest Dermatol 2011; 131:E8–11.

49 Mohl JE, Gerken T, Leung MY. Predicting mucin-type O-glycosylation using enhancement value products from derived protein features. J Thor Comp Med 2020; 19:2040003. https://doi.org/10.1142/s0219631620400039.

50 Wasinger C, Hofer A, Spaduti O et al. Amino acid signature in human melanoma cell lines from different disease stages. Sci Rep 2018; 8:6245.

51 Fu YM, Meadows GG. Specific amino acid dependency regulates the cellular behavior of melanoma. J Nutrition 2007; 117:1591S–6S; discussion 7S–8S.

© 2021 The Authors. British Journal of Dermatology published by John Wiley & Sons Ltd on behalf of British Association of Dermatologists British Journal of Dermatology (2022) 186, pp532–543

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.