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N-Glycosylation of extracellular matrix protein 1 (ECM1) regulates its secretion, which is unrelated to lipoid proteinosis

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ABSTRACT

Extracellular matrix protein 1 (ECM1) is expressed in a wide variety of tissues and plays important roles in extracellular matrix formation. Additionally, *ECM1* gene mutations cause lipoid proteinosis (LP), a rare skin condition of genetic origin. However, an effective therapeutic approach of LP is not established. Here, we showed that *ECM1* gene mutation observed in LP patients significantly suppresses its secretion. As ECM1 has three putative *N*-glycosylation sites and most of mutated ECM1 observed in LP patients are defective in these *N*-glycosylation sites, we investigated the correlation between LP and *N*-glycosylation of ECM1. We identified that the Asn^{354} and Asn^{444} residues in ECM1 were *N*-glycosylated by mass spectrometry analysis. In addition, an *N*-linked glycan at Asn^{354} negatively regulated secretion of ECM1, contrary to LP patient-derived mutants. These results indicate that the defect of *N*-glycosylation in ECM1 is not involved in the aberration of secretion of LP-derived mutated ECM1.

1. Introduction

Extracellular matrix protein 1 (ECM1) plays important roles in extracellular matrix formation, cell signaling, and regulation of differentiation in tissues [1]. ECM1 is a secretory protein initially isolated from an osteogenic stromal cell line and is constructed by a 19-amino-acid signal peptide and four functional domains: a cysteine-free N-terminal segment, two tandem repeats, and a C-terminal segment [2]. Several studies identified that ECM1 promotes angiogenesis, cell proliferation, and embryonal chondrogenesis [3–5]. Moreover, ECM1 inhibits matrix metalloproteinase 9 activity by high-affinity interaction [6]. Previous studies have shown that it also interacts with fibulin-1C/1D and perlecan, which are components of basement membrane protein, a thin sheet of fibers underlying the epithelium, including skin [7,8].

Additionally, an *ECM1* gene mutation causes lipoid proteinosis (LP), also known as Urbach–Wiethe disease, which is a rare autosomal recessive genodermatosis mainly observed in the Northern Cape province of South Africa [9]. LP is characterized by hoarseness because of infiltration of the vocal cords and thickening of the skin. Moreover, these patients show vascular anomalies caused by excessive deposition of hyaline-like material and consequent disruption/reduplication of basement membrane around blood vessels. It has been reported previously that exons 6 and 7 are the most common sites for ECM1 mutations in LP [10]. Since most of these mutations are nonsense mutations such as Q276X and W359X (Fig. 1A) or frameshift mutations, synthesis of full-length ECM1 is abolished. Although there have been many therapeutic trials in LP, including oral steroids and oral dimethyl sulfoxide (DMSO) [11,12], a definite therapeutic approach is not established.

Here, we focused on *N*-glycosylation in ECM1. Glycosylation is one of the post-translational modification reactions categorized into three types: *N*-glycosylation, *O*-glycosylation, and *C*-mannosylation. In the case of *N*-glycosylation, a glycan preassembled on a dolichol-phosphate carrier is transferred to Asn residues that are part of the consensus sequence Asn-Xaa-Ser/Thr (where Xaa can be any amino acid except Pro), and it is processed by a number of glycosidases and glycosyltransferases in the endoplasmic reticulum (ER) and the Golgi apparatus [13]. Previous report estimates that approximately 70–90% of the consensus sequence is glycosylated [14]. *N*-Glycosylation is involved in cellular processes, such as protein folding, intracellular trafficking, enzyme activity, and protein secretion [15]. Moreover, it is correlated with the pathogenesis

Abbreviations: ECM1, extracellular matrix protein 1; LP, lipoid proteinosis; ER, endoplasmic reticulum; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; CBB, Coomassie Brilliant Blue; PNGase F, peptide *N*-glycosidase F; MALDI-TOF MS, matrix-assisted laser desorption time flight mass spectrometry

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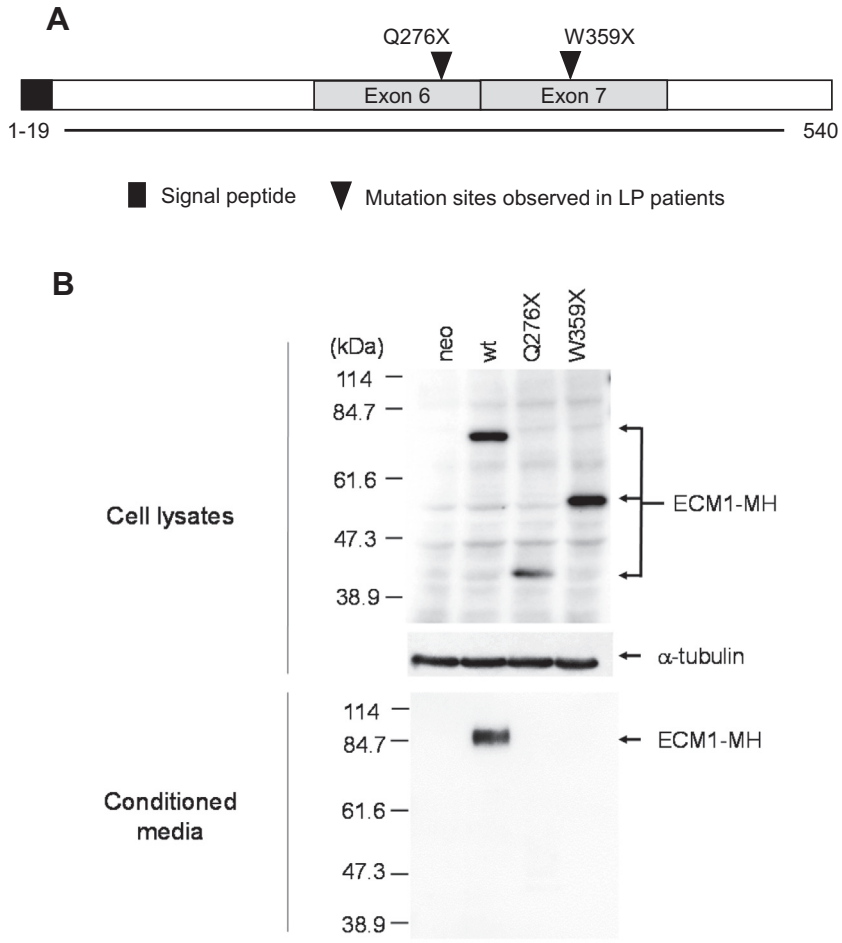

Fig. 1. Suppression of lipoid proteinosis-derived ECM1 secretion. (A) Schematic diagram of human ECM1 protein. ECM1 mutations observed in LP patients are indicated by the black inverted triangles. Signal peptide is indicated by the black box. (B) HT1080-neo, HT1080-ECM1-MH, HT1080-ECM1-Q276X-MH, and HT1080-ECM1-W359X-MH cells were cultured in serum-free media for 24 h. Subsequently, conditioned media and cell lysates were collected. Conditioned media were incubated with Ni-NTA agarose for 2 h at 4 °C. The bound proteins were eluted with 300 mM imidazole. Obtained samples were subjected to SDS–PAGE. The proteins were detected by immunoblotting with anti-c-myc or anti-α-tubulin antibodies.

of various diseases, including cancer [16,17]. *N*-Glycosylation has been reported to play various roles, but its roles seem to differ depending on the glycosylated protein.

In this study, we analyzed the correlation between LP and *N*-glycosylation of ECM1. ECM1 has putative *N*-glycosylation sites: Asn^{354}, Asn^{444}, and Asn^{530}, which exist in exon 7 and C-terminal domain. Since the mutations of *ECM1* frequently observed in LP patients are nonsense mutation in exons 6 and 7 [10], LP-derived mutated ECM1 is thought to be defective in *N*-glycosylation. Although some reports previously showed that ECM1 is *N*-glycosylated at Asn^{444} [18,19], the correlation between LP and *N*-glycosylation of ECM1 and the role of *N*-glycosylation on ECM1 remain unclear. This study identified all *N*-glycosylation sites in ECM1 and revealed that the defects in *N*-glycosylation in ECM1 are not responsible for the aberration in the secretion of LP-derived mutated ECM1.

2. Materials and methods

2.1. Cell culture

Human fibrosarcoma HT1080 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo, Japan), supplemented with 5% fetal bovine serum (FBS), 100 mg/L kanamycin, 100 units/mL penicillin G, 600 mg/L L-glutamine, and 2.25 g/L $NaHCO_3$ at 37 °C in a humidified incubator with 5% CO_2.

2.2. Construction of plasmids

The human C-terminally tagged ECM1-Myc-His_6 expression vector was generated by cloning human ECM1 cDNA from HL-60 cDNA into the pCI-neo vector. The c-myc and His_6 epitopes were fused after Pro^{275} (Q276X) or Thr^{358} (W359X) of human ECM1. The sequences of primers used for the mutagenesis were as follows: ECM1-Q276X-MH and ECM1-W359X-MH, 5′-TTTTCTCGAGATGGGGACCACAGCCAG-3′ (forward) and ECM1-Q276X-MH 5′-GATGAGTTTTTGTTCGGGAGCTTCCTCC-3′ (reverse); and ECM1-W359X-MH 5′-GATGAGTTTTTGTTCTGTACAGGTGTG-3′ (reverse).

The ECM1 N354Q-Myc-His_6 mutant, the ECM1 N444Q-Myc-His_6 mutant, and the N354Q/N444Q double mutant (designated as ECM1 2NQ-Myc-His_6) expression vectors were constructed by substituting Asn^{354} and/or Asn^{444} with Gln by using the overlap extension technique [20]. The sequences of primers used for the mutagenesis were as follows: ECM1-N354Q-MH, 5′-CGCCAGGGGAACCAACACACCTGTAC-3′ (forward) and 5′-GTACAGGTGTGTTGGTTCCCCTGGCG-3′ (reverse); ECM1-N444Q-MH, 5′-GGCTGATCCACCAAATGACTGCCCGC-3′ (forward) and 5′-GCGGGCAGTCATTTGGTGGATCAGCC-3′ (reverse).

2.3. Establishment of extracellular matrix protein 1-overexpressing cell lines

The stable cell lines expressing wild-type or mutant ECM1-myc-his$_6$ were established by transfecting the vectors into HT1080 cells and maintained in medium supplemented with 375 μg/mL G418 (Roche Applied Sciences, Indianapolis, IN). The stable cell lines that expressed high levels of myc-his$_6$-tagged wild-type ECM 1, ECM1 (Q276X), ECM1 (W359X), ECM1 (N354Q), ECM1 (N444Q), and ECM1 (2NQ; N354Q and N444Q) were designated as HT1080-ECM1-MH, HT1080-ECM1-Q276X-MH, and HT1080-ECM1-W359X-MH, HT1080-ECM1-N354Q-MH, HT1080-ECM1-N444Q-MH, and HT1080-ECM1-2NQ-MH cells, respectively. The cells transfected with pCI-neo were designated as HT1080-neo [21,22].

2.4. Western blot

Cells were cultured in 60-mm dishes with or without tunicamycin for 24 h, washed twice with phosphate-buffered saline (PBS), and lysed in a lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 1% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride) at 4 °C with sonication. The lysates were centrifuged at 14,000 rpm for 10 min at 4 °C, and the protein concentrations were determined by staining with Coomassie Brilliant Blue G-250 (Bio-Rad Laboratories, Hercules, CA). The samples were resolved using SDS–polyacrylamide gel electrophoresis (SDS–PAGE), transferred onto a PVDF membrane, immunoblotted with anti-c-myc (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-α-tubulin (Sigma–Aldrich, St. Louis, MO) antibodies, and visualized using an LAS 4000 mini (GE Healthcare, Waukesha, WI) [23–25].

2.5. Detection of secreted ECM1

The cells were cultured in serum-free media. After 24 h, the conditioned media and the cell lysates were collected. The conditioned media incubated with Ni-NTA agarose for 2 h at 4 °C. Ni-NTA agarose was washed with PBS, and Ni-NTA-bound ECM1 was eluted with 300 mM imidazole. The cell lysates were lysed as described above. The obtained samples were added with loading buffer (350 mM Tris–HCl, pH 6.8, 30% glycerol, 0.012% BPB, 6% SDS, 30% 2-ME) and boiled for 3 min. Secondly, the samples were resolved using SDS–PAGE, transferred onto a PVDF membrane, and immunoblotted with anti-c-myc or anti-α-tubulin antibodies [26].

2.6. Purification of recombinant extracellular matrix protein 1 from cell culture medium

HT1080-ECM1-MH cells were cultured in serum-free medium for 24 h. After 24 h, conditioned medium was collected and incubated with Ni-NTA agarose for 2 h at 4 °C. Ni-NTA agarose (Qiagen, Hilden, Germany) was washed with PBS, and Ni-NTA-bound ECM1 was eluted with 300 mM imidazole. The obtained samples were subjected to SDS–PAGE and stained with Coomassie Brilliant Blue R-250 [27–29].

2.7. Liquid chromatography–mass spectrometry

Recombinant ECM1 purified from conditioned media was denatured using 1% SDS at 95 °C for 5 min and was diluted 1:10 with 1% Triton X-100. To deglycosylate the denatured protein, the sample was treated using peptide *N*-glycosidase F (PNGaseF; Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer recommendations. Both deglycosylated and untreated samples were subjected to SDS–PAGE. After CBB staining, a visible band was excised and destained. In-gel digestion was performed using trypsin (TPCK-treated, Worthington Biochem. Co., Freehold, NJ). The digestion mixture was separated using a nanoflow LC (Easy nLC, Thermo Fisher Scientific, Waltham, MA) on a PepMap RSLC analytical column (C18, φ50 μm × 15 cm, 2 μm, 100 Å, Thermo Fisher Scientific) with a linear gradient of 0–35% buffer B (100% ACN and 0.1% formic acid) at a flow rate of 300 nL/min over 10 min and subjected on-line to a Q Exactive mass spectrometer (Thermo Fisher Scientific) with a nanospray ion source. MS and MS/MS data were acquired using a data-dependent top5 method. Obtained MS/MS data were searched against an in-house database, including the ECM1 sequence, using the MASCOT program (Matrix Science, London, UK) with variable modifications: Gln → pyro-Glu (N-term Q), Oxidation (M), Propionamide (C), Hex (W).

2.8. MALDI-TOF MS

Purified recombinant ECM1 was subjected to SDS–polyacrylamide gels. After CBB staining, the bands were excised with trypsin (TPCK-treated, Worthington Biochem. Co.). The digests were desalted using ZipTip C18μ tips (EMD Millipore Co., Billerica, MA) and applied to MALDI-TOF MS on an ultrafleXtreme TOF/TOF MS (Bruker Daltonics, Bremen, Germany) in reflector mode using α-cyano-4-hydroxycinnamic acid as the matrix. The selected peaks were analyzed by MS/MS in LIFT mode.

3. Results

3.1. Suppression of lipoid proteinosis-derived ECM1 protein secretion

Previous reports have shown that ECM1 mutations are frequently observed in LP patients and clinical features caused by ECM1 mutations have been well investigated. However, the underlying mechanisms by which ECM1 mutations cause LP have not been analyzed. Therefore, we analyzed the phenotypes of LP-derived mutant ECM1-overexpressing cells compared with wild-type ECM1-overexpressing cells.

First, we established ECM1-overexpressing HT1080 cell lines (HT1080-ECM1-MH) and established cell lines overexpressing mutant forms of ECM1 observed in LP patients (Q276X and W359X) in HT1080 cells. Subsequently, we tried to examine the secretion levels of two ECM1 mutants. We investigated the levels of secreted ECM1 from HT1080-neo, HT1080-ECM1-MH, HT1080-ECM1-Q276X-MH, and HT1080-ECM1-W359X-MH cells. As a result, secreted Q276X and W359X were not observed (Fig. 1B), suggesting that aberration of ECM1 secretion is one of the causes of LP. We revealed for the first time that LP-derived mutant ECM1 was less secreted compared with wild-type ECM1.

3.2. ECM1 is N-glycosylated at Asn^{354} and Asn^{444} residues

Previous report showed that nonsense mutations in exons 6 and 7 of ECM1 are frequently observed in LP patients [10]. Moreover, three putative *N*-glycosylation sites in ECM1 exist between exon 7 and the C-terminal domain (Fig. 2A). Thus, *N*-linked glycan cannot attach to mutated ECM1 observed in LP patients. Furthermore, some studies have reported that *N*-linked glycan is important for protein secretion [21,22,30–32]. For these reasons, we hypothesized that the aberrant *N*-glycosylation by gene mutation in *ECM1* is one of the causes of LP. To validate this hypothesis, we analyzed *N*-glycosylation of ECM1.

First, we examined whether ECM1 is *N*-glycosylated or not. We treated HT1080-neo and HT1080-ECM1-MH cells with tunicamycin, an inhibitor of protein *N*-glycosylation. Our data showed that treatment of HT1080-ECM1-MH cells with tunicamycin led to a

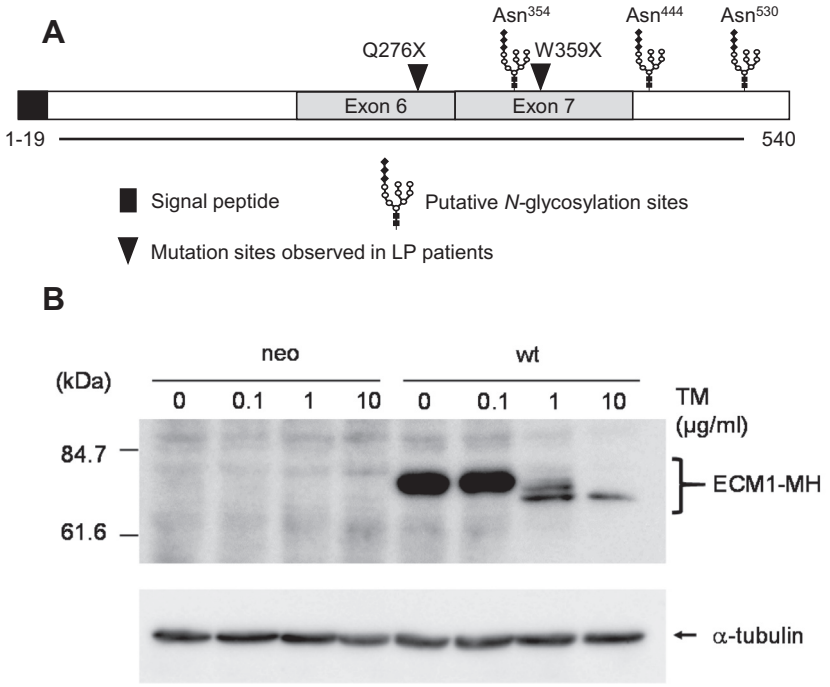

Fig. 2. Treatment of tunicamycin suggested *N*-glycosylation within ECM1. (A) Schematic diagram of human ECM1 protein. ECM1 mutations observed in LP patients are indicated by the black inverted triangles. Three putative *N*-glycosylation sites (Asn^{354}, Asn^{444} and Asn^{530}) are indicated by sugar chains. Signal peptide is indicated by the black box. (B) HT1080-neo and HT1080-ECM1-MH cells were treated with tunicamycin (TM) at various concentrations (0, 0.1, 1, and 10 μg/mL) for 24 h. The cells were lysed, and aliquots of the cell lysates were subjected to SDS–PAGE. The proteins were detected by immunoblotting with anti-c-myc or anti-α-tubulin antibodies.

size reduction of ECM1-MH (Fig. 2B), suggesting that ECM1 was *N*-glycosylated.

Next, we performed mass spectrometry analysis to determine the *N*-glycosylation sites in ECM1. Recombinant ECM1 was purified from conditioned medium of HT1080-ECM1-MH cells using Ni-NTA agarose (Fig. 3A). Purified recombinant ECM1 was digested with trypsin and was treated with or without PNGase F. The resulting peptides were analyzed by MALDI-TOF MS or LC–MS. LC–MS was used when the peptide fragment including putative *N*-glycosylated Asn residue could not be detected by MALDI-TOF MS. Because PNGase F cleaves between the innermost GlcNAc and Asn residues of *N*-linked glycans, thereby converting Asn to Asp residues, the new peaks of the fragment showing conversion of glycosylated Asn to Asp can be observed by mass spectrometry analysis. With PNGase F treatment, the peptide fragment including Asp that was converted from Asn^{354} residue was observed at b3 (283.10) to b5 (535.19) by LC–MS/MS (Fig. 3B). Similarly, with PNGase F treatment, the peptide fragment including Asp that was converted from Asn^{444} residue was observed at *m*/*z* 1360.7 by MALDI-TOF MS (Fig. 3C). However, the peak of the fragment including Asn^{530} was detected by LC–MS, even though the sample was treated with or without PNGase F (Fig. 3D). Moreover, the peak of the fragment including Asp that was converted from Asn^{530} residue was not observed (Fig. 3D). These results indicate that the Asn^{354} and Asn^{444} residues, but not Asn^{530}, are *N*-glycosylated.

To further confirm the *N*-glycosylation of ECM1, we prepared Asn-to-Gln single and double mutants corresponding to *N*-glycosylated Asn residues and established cell lines overexpressing mutant forms of ECM1 (N354Q, N444Q, and 2NQ) in HT1080 cell lines (Fig. 4A). Western blotting of these stable cell lines revealed that the molecular size of the single mutant was lower than that of wild-type ECM1 and that the molecular size of the double mutant (2NQ) was lower than that of the single mutant (N354Q and N444Q) (Fig. 4A). Subsequently, we treated HT1080-ECM1-MH and HT1080-ECM1-2NQ-MH cells with tunicamycin. Treatment with tunicamycin led to a size reduction of wild-type ECM1 but not 2NQ (Fig. 4B). Moreover, the molecular size between wild-type ECM1 treated with tunicamycin and 2NQ was the same. These results indicate that ECM1 is *N*-glycosylated at the Asn^{354} and Asn^{444} residues.

3.3. N-Glycosylation regulates secretion of ECM1

Since the secretion levels of ECM1 mutants (Q276X and W359X) observed in LP patients were suppressed, we investigated the role of *N*-glycosylation for secretion of ECM1 to elucidate a link between LP and *N*-glycosylation of ECM1.

Our data showed that the levels of secreted ECM1-N354Q-MH and ECM1-2NQ-MH mutants were higher than that of wild-type ECM1. On the other hand, the levels of secreted wild-type ECM1-MH and ECM1 N444Q-MH were approximately the same (Fig. 4C). These results indicate that *N*-glycosylation at Asn^{354} negatively regulates the secretion of ECM1, whereas *N*-glycosylation at Asn^{444} has no effect on the secretion. Since *ECM1* gene mutation observed in LP patients (Q276X and W359X) significantly suppresses its secretion, the aberrant *N*-glycosylation in ECM1 does not seem to be the cause of LP directly.

4. Discussion

ECM1 plays important roles in cell proliferation, angiogenesis, and extracellular matrix formation [1,4]. Mutations in the *ECM1* gene are identified in patients of skin disease, LP, for which an effective treatment has not be established [11]. We examined the effect of ECM1 mutations that are observed in LP patients (Q276X and W359X) on its secretion and revealed for the first time that the levels of each secreted mutant were significantly lower than that of wild-type ECM1 (Fig. 1B), suggesting that aberration of ECM1 secretion is one of the causes of LP.

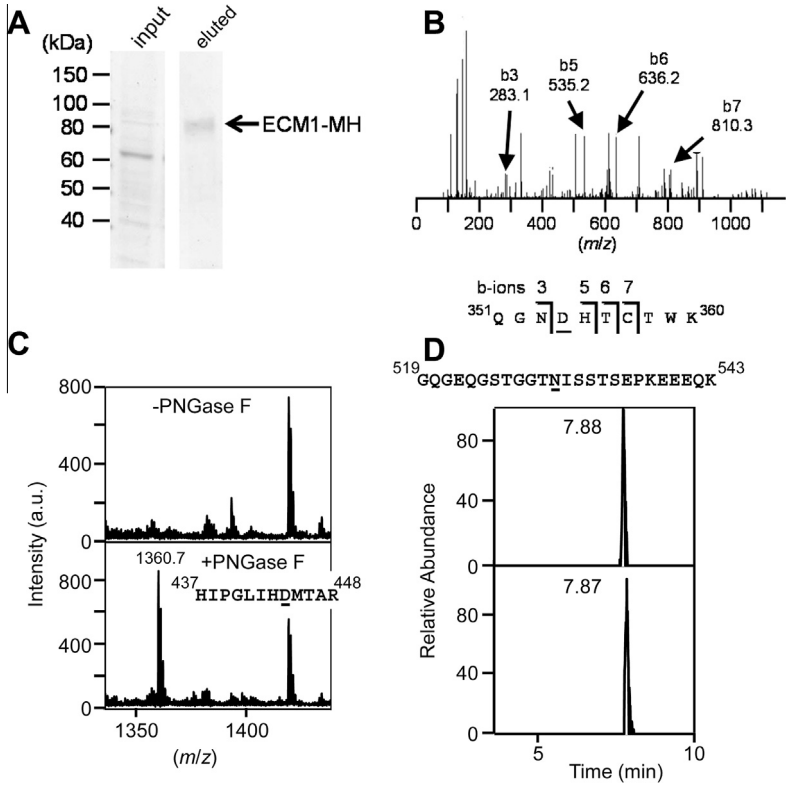

Fig. 3. *N*-Glycosylation of human ECM1 at Asn^{354} and Asn^{444}. (A) HT1080-ECM1-MH cells were cultured in serum-free medium for 24 h. Subsequently, conditioned medium was collected and incubated with Ni-NTA agarose for 2 h at 4 °C. The bound proteins were eluted with 300 mM imidazole, subjected to SDS–PAGE, and detected by CBB staining. (B) Purified recombinant ECM1 was treated with PNGaseF and subjected to SDS–PAGE. The sample that was digested with trypsin was analyzed by LC–MS/MS. Asn^{354} was converted to Asp residue (underlined "D") by treatment with PNGaseF. (C) Purified ECM1 was treated with (lower) or without (upper) PNGaseF and subjected to SDS–PAGE. The samples that were digested with trypsin were analyzed by MALDI-TOF MS. The peak of the fragment in which Asn^{444} was converted to Asp residue (underlined "D") by PNGaseF was observed at *m*/*z* 1360.7. (D) Purified ECM1 was treated with (lower) or without (upper) PNGaseF and subjected to SDS–PAGE. The samples that were digested with trypsin were analyzed by LC–MS. The peak of the fragment including Asn^{530} was observed at 7.8 min both after treatment with or without PNGaseF.

N-Glycosylation is a common post-translational modification reaction that attaches an *N*-linked glycan to an Asn residue, which is a part of the consensus sequence. It mostly participates in protein folding, intracellular trafficking, enzyme activity, and protein secretion [15]. Previous reports have shown that ECM1 is *N*-glycosylated at Asn^{444} [18,19]. However, the underlying biological functions of glycosylation in ECM1 are yet to be reported. In this study, we studied the correlation between LP and *N*-glycosylation through identification of the all *N*-glycosylation sites in ECM1.

We demonstrated that ECM1 is *N*-glycosylated at Asn^{354} and Asn^{444} by mass spectrometry analysis (Fig. 3B and C). This is the first report identifying the *N*-glycosylation sites of ECM1 completely. Moreover, we demonstrated that *N*-glycosylation at Asn^{354} negatively regulates the secretion of ECM1 (Fig. 4C). It has been reported that the secretion of many *N*-glycosylated proteins is positively regulated by their glycosylation, contrary to ECM1 [21,22,30–32]. Although, further studies are needed to know why *N*-glycosylation of ECM1 negatively regulates its secretion, we speculated that an *N*-linked glycan at Asn^{354} might be recognized by some factors, thereby suppressing the secretion of ECM1. Based on our observations, the secreted levels of ECM1 mutants observed in LP patients were lower than that of wild-type ECM1, contrary to *N*-glycosylation-defective mutants. Thus, contrary to our hypothesis, we conclude that aberrant *N*-glycosylation is not the cause of LP. We speculated that suppression of LP mutants secretion might be attributed to conformational changes of ECM1 by gene mutations observed in LP patients, thereby allowing it to be degraded by ER-associated degradation. Besides, the skin of LP patients show hyperkeratosis and basement membrane thickening at the dermal-epidermal junction [1]. Possibly, these phenotypes may result from the reduction of secreted levels of ECM1 in LP patients. However, the possibility cannot be excluded that the *N*-glycosylation patterns may be different between *in vitro* and in human tissues because we evaluated using ECM1-overexpressing cell lines in this study.

Meanwhile, ECM1 has a putative *C*-mannosylation site at Trp^{359} (the consensus sequence is Trp-Xaa-Xaa-Trp/Cys; Trp^{359}-Lys-Ala-Trp). *C*-mannosylation is a unique type of glycosylation that covalently attaches an α-mannopyranosyl residue to the indole C2 carbon atom of tryptophan via a C–C linkage [33]. We also analyzed whether ECM1 is *C*-mannosylated by mass spectrometry and revealed that ECM1 was not *C*-mannosylated (data not shown). On the other hand, we demonstrated that ECM1 is *O*-glycosylated at Ser^{525} and/or Thr^{526} (data not shown). *O*-glycosylation attaches a sugar chain to an oxygen atom of serine/threonine and seems to influence proteolytic processing and protein folding [34]. We intend to analyze the roles of *O*-glycosylation on ECM1 and its relationship to LP in future work.

In conclusion, we identified that the *ECM1* gene mutation observed in LP patients significantly suppresses its secretion. Additionally, we revealed that ECM1 is *N*-glycosylated at two sites, Asn^{354} and Asn^{444} and *N*-glycosylation at Asn^{354} negatively regulates the secretion of ECM1. These results indicate that LP is unrelated to *N*-glycosylation of ECM1. Our results contribute to the understanding of the mechanisms of LP and provide new insights into ECM1 function.

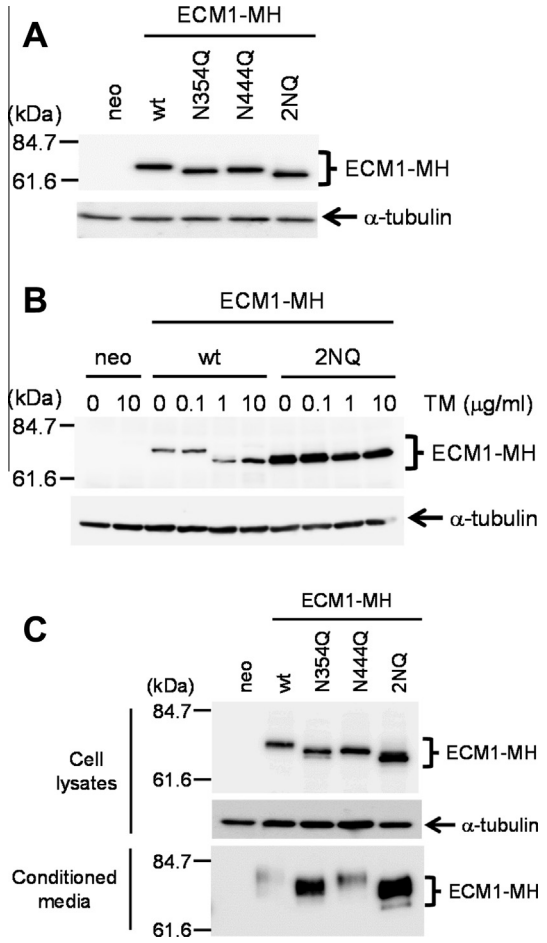

Fig. 4. Negative regulation of ECM1 secretion by *N*-glycosylation at Asn[354]. (A) HT1080-neo, HT1080-ECM1-MH, HT1080-ECM1-N354Q-MH, HT1080-ECM1-N444Q-MH, and HT1080-ECM1-2NQ-MH cells were lysed, and aliquots of the cell lysates were subjected to SDS–PAGE. The proteins were detected by immunoblotting with anti-c-myc or anti-α-tubulin antibodies. (B) HT1080-neo, HT1080-ECM1-MH, and HT1080-ECM1-2NQ-MH cells were treated with tunicamycin (TM) at various concentrations (0, 0.1, 1, and 10 μg/mL) for 24 h. The cells were lysed, and aliquots of the cell lysates were subjected to SDS–PAGE. The proteins were detected by immunoblotting with anti-c-myc or anti-α-tubulin antibodies. (C) HT1080-neo, HT1080-ECM1-MH, HT1080-ECM1-N354Q-MH, HT1080-ECM1-N444Q-MH, and HT1080-ECM1-2NQ-MH cells were cultured in serum-free media for 24 h. Subsequent conditioned media and cell lysates were collected. Conditioned media were incubated with Ni-NTA agarose for 2 h at 4 °C. The bound proteins were eluted with 300 mM imidazole. Obtained samples were subjected to SDS–PAGE. The proteins were detected by immunoblotting with anti-c-myc or anti-α-tubulin antibodies.

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