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Context-dependent regulation of collagen XVII ectodomain shedding in skin

Short running head:

Regulation of COL17 ectodomain shedding

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Abstract:

Pemphigoid is a common autoimmune blistering disorder in which autoantibodies target transmembrane collagen XVII (COL17), a component of hemidesmosomes in basal keratinocytes. The ectodomain of COL17 can be cleaved from the cell surface within the juxtamembranous extracellular NC16A domain, and interestingly, certain autoantibodies of pemphigoid patients preferentially react with the shed ectodomain. These findings suggest that COL17 ectodomain shedding generates neoepitopes on the shed form; however, the regulatory mechanism of the shedding in *in vivo* skin and the pathogenicity of the neoepitope-targeting antibodies are still uncertain. To address these issues, we produced rabbit antibodies specifically reacting with N-terminal cleavage sites of the shed COL17 ectodomain. The antibodies revealed that certain amounts of human COL17 ectodomain is physiologically cleaved at Gln^{525} in *in vivo* skin. In contrast, migrating human keratinocytes cleave COL17 at Leu^{524} but not at Gln^{525}. Passive transfer of antibodies reacting with an N-terminal cleavage site of mouse COL17 ectodomain into neonatal wild-type mice failed to induce blister formation, even though the antibodies bound to the dermal-epidermal junctions, indicating that cleavage-site-specific antibodies have reduced or absent pathogenicity for blister formation. This study shows the ectodomain shedding of COL17 to be a physiological event in *in vivo* human skin that probably generates non-pathological epitopes on the cleavage sites.

Bullous pemphigoid (BP), a major pemphigoid subtype, is a common autoimmune blistering skin disease in which autoantibodies (autoAbs) target two hemidesmosomal components: transmembrane collagen XVII (COL17)/BP180 and the plakin family protein BP230 [1-5]. Previous studies have demonstrated that COL17 is the major autoantigen (autoAg) not only for BP but also for other pemphigoid disorders, including linear IgA bullous dermatosis (LAD), mucous membrane pemphigoid, pemphigoid gestationis and lichen planus pemphigoides [3]. The major epitopes for BP autoAbs tightly cluster within the juxtamembranous non-collagenous NC16A domain of COL17, and the majority of IgG autoAbs react with this region [6, 7]. Passive transfer experiments of rabbit IgG [8] and autoAbs from BP patients [9] into wild-type (WT) or transgenic (Tg) neonatal mice have demonstrated pathogenic roles of antibodies (Abs) to the NC16A domain in blister formation.

COL17 is a type II-oriented transmembrane glycoprotein whose N-terminus and C-terminus are in the cytoplasm and the extracellular matrix (ECM), respectively [10, 11]. As is true for other transmembrane collagen family members, the extracellular domain of COL17 can be cleaved from the cell surface *in vitro* by a disintegrin and metalloproteases (ADAMs) 9, 10 and 17 [12, 13], and interestingly, the ectodomain shedding occurs within the NC16A domain which contains major epitopes for BP autoAbs [14, 15]. In addition, it is known that autoAbs from LAD [16] and certain BP patients [17] preferentially react with the shed ectodomain but not with the full-length form of COL17. This unique characteristic was also observed

in several monoclonal Abs (mAbs) directing the ectodomain of COL17 [11, 18-20], suggesting that neoepitopes appeared on the shed ectodomain of COL17. Among them, mAb 123, which specifically reacts with the shed COL17 ectodomain, has been shown to induce subepidermal blister formation on unfixed human frozen skin *in vitro* [18]. However, the precise pathomechanism for the generation of neoepitopes after COL17 cleavage has not been elucidated.

Recently, we have identified physiological N-terminal cleavage sites of human COL17 within the NC16A domain *in vitro*: Asp^{514}, Leu^{524}, Gln^{525} and Gly^{526} [15]. In addition, rabbit IgG Abs targeting the cleavage site of Leu^{524} (Ab HK139) specifically react with the shed COL17 ectodomain, indicating that the cleavage of the molecule actually generates neoepitopes. Furthermore, incubation of cryosections of normal human skin (NHS), neutrophils and Ab HK139 induced dermal-epidermal separation *in vitro*, suggesting pathogenic roles for the neoepitope-specific Abs in blister formation [15]. However, the Ab HK139 showed mottled staining at the dermal-epidermal junction (DEJ) of NHS; thus, Leu^{524} may be a minor cleavage site in a physiological setting and a different cleavage site or sites may be involved *in vivo*. In addition, it is still uncertain whether neoepitope-specific Abs to COL17 are pathogenic for blister formation *in vivo*.

In this study, we tried to determine whether COL17 is physiologically cleaved and whether the ectodomain shedding of COL17 produces neoepitopes in *in vivo* skin, and we investigated the pathogenic roles of Abs targeting the neoepitopes at the cleavage sites in blister formation. It was found that novel Abs targeting a

physiological cleavage site demonstrated that the ectodomain of human COL17 is able to be shed and that cleaved ectodomain is present at the DEJ even in a physiological steady setting. In contrast, the cleavage site or sites of COL17 ectodomain are differentially targeted when keratinocytes migrate in *in vitro* cultured keratinocytes. Finally, passive-transfer experiments using Abs targeting a candidate N-terminal physiological cleavage site of the mouse COL17 ectodomain failed to induce blister formation in mice, suggesting that the immune reaction to the neoepitopes on the physiological cleavage sites is not pathogenic.

Materials and methods

Cell Culture

Primary normal human epidermal keratinocytes (NHEKs) isolated from neonatal foreskin (Lonza) were cultured in keratinocyte growth medium (KGM, Lonza). To isolate primary mouse keratinocytes (NMKs), neonatal WT mice on a C57BL/6J background (Clea) were sacrificed, and whole skin sections were incubated with 0.25% trypsin EDTA (Gibco) for 1 h at 37°C. After the epidermal layer was scraped and incubated with 10% FCS in PBS (Gibco), primary NMKs were seeded on plastic with Cnt-57 medium supplemented with bovine pituitary extract (Cellntec). Cells up to the 4th passage were used.

Generation of Abs

Rabbit polyclonal Abs targeting the N-terminal cleavage site Gln^{525} of human COL17 (Ab Hu-pyro-Glu) were produced by immunizing two New Zealand white

rabbits with peptides corresponding to 8 amino acids (aa) [15]. Since N-terminal Gln^{525} is physiologically cyclized to pyroglutamate (pyro-Glu) *in vitro* [15], the peptides pyro-Glu-Gly-Met-Ala-Pro-Ala-Ala-Gly-Cys were chemically synthesized using L-pyroglutamic acid (Hokudo). The peptides were coupled with keyhole limpet hemocyanin through carboxyl terminal Cys with m-Maleimidobenzoyl-N-hydroxysuccinimide ester [15]. Whole IgG from the immunized rabbit serum was first affinity-purified with protein A Sepharose (ProteNova) according to the manufacture's protocol. A portion of the whole IgG Abs were immunoadsorbed using chemically synthesized R7 peptide (Asp^{522} to Gln^{545} of human COL17, Hokudo) to eliminate Abs that react with the full-length COL17. Briefly, whole IgG Abs were dialyzed to PBS, followed by incubation with R7 peptide-coupled NHS-activated HP (GE Healthcare), as we previously performed [15, 21]. Flow-through samples containing unbound Abs were corrected, and the concentration was measured using the BCA protein assay kit (Thermo Fisher Scientific). The immune adsorbed Ab Hu-pyro-Glu was used for experiments in this study not otherwise specified. Rabbit Abs against the NC16A domain (Glu^{490} to Arg^{566} of human COL17, Ab Hu-NC16A) [21] targeting the cleavage site Leu^{524} (Ab HK139) [15] and the C-terminus (Val^{1192} to Pro^{1497} of human COL17, Ab 09040) were produced as previously described [15].

Rabbit polyclonal Abs targeting a candidate N-terminal cleavage site of mouse COL17, Gln^{532} (Ab Mo-pyro-Glu), were produced by immunizing rabbits with synthetic peptides pyro-Glu-Ala-Glu-Ala-Pro-Ser-Leu-Gly-Cys (Hokudo). The whole Abs were first prepared by using protein A Sepharose as described above,

and they were further affinity-purified with antigenic peptides by using the SulfoLink Immobilization Kit (Thermo Fisher Scientific) according to manufacturer's instruction. Precise information about epitopes for these Abs are illustrated in **Figure 1**. To produce rabbit Abs against the mouse NC14A domain of COL17 (Ab Mo-NC14A), GST-conjugated mouse NC14A protein (Mo-NC14A) was prepared as previously described [9]. Briefly, PCR products encoding 75-aa NC14A polypeptides (from Glu^{499} to Arg^{573} of mouse COL17) were amplified by using the primers mNC14A-F (5'-CCGAATTCGAGGAGGTAAGAAAGCTGAA-3'; the EcoRI restriction site is underlined) and mNC14A-R (5'-GGGCGGCCGCTCTGAGATTCCCGTTCTCCT-3'; the NotI restriction site is underlined), and cDNA from mouse keratinocytes. The 243-bp PCR product was digested by EcoRI and NotI, followed by integration into pGEX-6P-1 vector (GE Healthcare). After DNA sequences were confirmed, the expression vector was transformed into *E. coli* (BL21, GE Healthcare). The cells were lysed by B-PER protein extraction reagent (Thermo Fisher Scientific), and GST-Mo-NC14A protein was purified by GSTrap affinity column (GE Healthcare) according to the manufacturer's instructions. Correct expression of Mo-NC14A as a GST-fusion protein was confirmed by digesting it with PreScission Protease (GE Healthcare). Similarly, recombinant mouse NC14A protein with C-terminal hexahistidine and HSV tag sequences (Mo-NC14A-His·HSV) was produced using the same primers and the pET-44a (+) vector (Novagen). For the control, an empty vector was used. To obtain the Ab Mo-NC14A, four New Zealand white rabbits were immunized with the purified Mo-NC14A protein, and the whole IgG was affinity-purified as mentioned above. The specificity of the Ab Mo-NC14A to the

NC14A domain of mouse COL17 was assessed by immunoblotting using Mo-NC14A-His·HSV protein.

Immunoblotting and Immunohistochemistry

For immunoblotting, subconfluent NHEKs or NMKs were lysed for 30 min on ice in a buffer 1% Nonidet P-40, 0.1 M NaCl, 25 mM Tris-HCl, pH 7.4, 10 mM EDTA, 1 mM Pefabloc SC (Sigma) [12]. Medium proteins were precipitated by ethanol [12]. To address spontaneous cyclization of N-terminal Gln^{525} into pyro-Glu in human COL17 ectodomain, sterilized culture medium of NHEKs was incubated at 37°C for 2, 4, 9 days respectively before the ethanol precipitation. Preparation of the ECM proteins was performed as described elsewhere [22, 23]. Briefly, the cell layer was incubated at room temperature with 20-mM NH_4OH solution after being washed with PBS. After all the cells had detached, the ECM was extensively washed with PBS and dissolved in SDS-PAGE sample buffer. The samples were separated on SDS-PAGE 7% polyacrylamide gels, followed by transfer onto nitrocellulose membrane. The following Abs were used: anti-HSV-tag Ab (A00624, Genscript), the rabbit polyclonal Abs described above and mouse mAb NC16A-3 directing NC16A domain of human COL17 (provided by Prof. Bruckner-Tuderman). After incubation with an HRP-conjugated secondary Ab, signals were visualized by ECL-plus (GE Healthcare). For indirect immunofluorescence (IIF) microscopy, skin specimens of NHS, skin from a COL17-lacking epidermolysis bullosa patient [24], normal mouse skin (NMS), *mCol17*$^{-/-}$ COL17-lacking mice skin, COL17-humanized (*mCol17*$^{-/-}$*, hCOL17*$^{+/+}$) mice skin [9], and NHEKs cultured on cover slips for 18 hrs were used. For IIF of

wound skin, 3 samples of postoperative skin ulcers taken from a 26-year-old man, a 68-year-old man and a 69-year-old man were used, from whom pilomatricoma on the left upper arm, the left inguinal lymph nodes and malignant melanoma on the left sole had been surgically removed 25, 37 and 35 days before, respectively. For some experiments, human or mouse skin sections were incubated with 1 M NaCl to produce artificial blisters for 14 and 24 hrs at 4ºC, respectively. The skin specimens were mounted and snap-frozen in optimal cutting temperature compound (Tissue-Tek O.C.T., Sakura), and 5-µm cryosections were incubated with antiserum, purified rabbit polyclonal IgG Abs or serum from rabbit Ab-injected mice for 1 h at room temperature and detected with 1:1,000 diluted Alexa488-conjugated anti-rabbit IgG (Invitrogen) and propidium iodide (PI, Wako). For direct immunofluorescence (DIF) microscopy, Alexa488-conjugated anti-rabbit IgG and FITC-conjugated anti-mouse C3 (Cappel) were used. IIF on cultured NHEKs was performed using rhodamine phalloidin (Invitrogen) as previously described [23], and images were observed by fluorescence microscopy (IX-51, Olympus) or confocal microscope (FX81, Olympus).

Immunoprecipitation (IP)

IP was performed using rabbit polyclonal Abs as described above and normal rabbit polyclonal IgG (37415, Abcam) as a control, cell lysates of cultured NHEKs prepared as described, and culture medium. Briefly, 50 µl of protein-G Sepharose (GE Healthcare) was coupled with 50 µg of Abs Hu-pyro-Glu, Hu-NC16A or normal rabbit IgG, followed by incubation with cell lysates of NHEKs or 1ml of culture medium for 1 hr at room temperature. Ab Hu-pyro-Glu After extensive

washing of the beads with PBS containing with 0.1% NP-40, bound proteins were eluted by boiling for 5 min with 1x SDS sample buffer. To assess whether N-terminus of shed human COL17 ectodomain is cyclized from Gln^{525} into pyro-Glu or not, the 3ml of culture medium of NHEKs was concentrated and exchanged buffer to 25mM HEPES, pH7.0 by Amicon Ultra Centrifuge Filter Unit (30kD, Millipore). The concentrated culture medium was treated with 1μg of recombinant human glutaminyl-peptide cyclotranferase (6368-ZN, R&D Systems) for 24hrs at 37°C following by IP using Ab Hu-pyro-Glu. TruBlot (Rockland Immunochemicals) was used as a secondary Ab.

Passive Transfer of the Cleavage-Specific Abs into Mice

Neonatal and 8-wk-old WT C57BL/6J mice were used. The neonatal mice received a single intraperitoneal injection of 250 μg/g body weight of the Ab Mo-pyro-Glu (n=6), which had been affinity-purified with peptide beads, or 2,000 μg/g body weight of whole IgG containing the Ab Mo-NC14A (n=4). Forty-eight hrs after the injection, back skin was separately evaluated by two investigators for detachment by gentle rubbing [9, 21]. For adult mice, 500 μl of antiserum containing the Ab Mo-pyro-Glu was subcutaneously injected each day for 5 consecutive days, and skin disease was assessed 3 days after the last injection (n=4). For the control, pre-immune serum or normal whole rabbit IgG (Abcam) was used.

All mouse procedures were approved by the Institutional Animal Care and Use Committee of Hokkaido University. This study was approved by the Ethical Committee of Hokkaido University, and fully informed consent was obtained from

all patients and healthy volunteers for the use of their materials.

Results

Gln^{525} of COL17 is a highly conserved, physiological N-terminal cleavage site in normal human skin

We previously reported that different cleavage sites cluster within the NC16A domain of COL17 (**Fig. 1A**) [15]. Although amino acids sequences covering the NC16A domain show significant differences among different species [10, 25], the cleavage site Gln^{525} is highly conserved (**Fig. 1B**), indicating that it is a functionally important amino acid. Interestingly, our previous studies showed that the N-terminal Gln^{525} is unique, since its N-terminus is naturally cyclized by changing from Gln into pyro-Glu after cleavage *in vitro* [15]. To assess whether human COL17 is actually cleaved at this site and whether the N-terminus was changed from Gln^{525} to pyro-Glu^{525} *in vivo*, we produced rabbit Abs (Ab Hu-pyro-Glu) targeting the modified cleavage site (**Fig. 1A**). Western blotting showed that the Ab Hu-pyro-Glu weekly reacts with the 120-kD shed ectodomain and not with the 180-kD full-length form of COL17 (**Fig. 2A**). However, IP studies using high doses of the Ab and plenty of NHEK cell lysate revealed that the Ab was able to immunoprecipitate small amounts of full-length COL17 (**Fig. 2B**). Therefore we immunoadsorbed the Ab using synthesized R7 peptide (Asp^{522} to Gln^{545}) [21], which does not contain Gln^{525} as an N-terminal cleavage site, and the treatment eliminated the reaction to full-length COL17 (**Fig. 2B**). The ability of Ab Hu-pyro-Glu to immunoprecipitate 120kD COL17 ectodomain was also

disappeared after the immunoadsorption (**Fig. 2B**), indicating shed COL17 ectodomain with pyro-Glu N-terminus is not present or small amounts in culture medium of NHEKs. Immunoreactivity of the Ab Hu-pyro-Glu to NHS was not reduced after immunoadsorption (**Fig. 2C**), thus we speculated that N-terminal cyclization from Gln^{525} into pyro-Glu of the shed molecule may preferentially occur *in vivo* and that culture medium of NHEKs contains shed COL17 ectodomain with non-cyclized N-terminal Gln^{525}. To address these hypotheses, we treated culture medium of NHEKs with recombinant human glutaminyl-peptide cyclotransferase, an enzyme which cyclizes N-terminal Gln into pyro-Glu. The treatment produced larger amounts of shed COL17 ectodomain which is targeted by Ab Hu-pyro-Glu (**Fig. 2D**), confirming presence of shed COL17 ectodomain with non-cyclized N-terminal Gln^{525}. In addition, the Ab Hu-pyro-Glu precipitated increased amounts of COL17 ectodomain from NHEKs culture medium treated by glutaminyl-peptide cyclotransferase (**Fig. 2D**), proving specific binding to COL17 ectodomain with pyro-Glu N-terminus. Furthermore, spontaneous cyclization of N-terminal Gln^{525} in culture medium incubated at 37°C was confirmed (**Fig. 2E**). These findings proved that majority of COL17 ectodomain in culture medium of NHEKs has non-cyclized Gln^{525} N-terminus and the Ab Hu-pyro-Glu specifically reacts with pyro-Glu N-terminal shed COL17 ectodomain. The immune adsorbed Ab Hu-pyro-Glu failed to react with skin sections obtained from an epidermolysis bullosa patient lacking COL17 (**Fig. 2F**). This confirmed that the reaction was specific to human COL17. Interestingly, the Ab Hu-pyro-Glu showed positive staining on the epidermal side of artificial blisters of the NHS induced by pre-incubation with 1-M NaCl for 14 hrs

(**Fig. 2F**), suggesting the presence of an unknown binding molecule or molecules that anchors the shed COL17 ectodomain in the ECM. The reactivity of the Abs to the epidermal side was diminished when the incubation time of NHS with 1-M NaCl was increased to 24 hrs to induce artificial blisters (not shown), indicating that epitopes of the Ab Hu-pyro-Glu are probably cleaved off by internal proteases.

COL17 ectodomain shedding in migrating keratinocytes is differentially regulated in in vitro and in vivo

We previously showed that migrating NHEKs cleave COL17 at Leu^{524} and that the cleaved ectodomain of COL17 is detectable by the Ab HK139 targeting this cleavage site as migration tracks in the ECM *in vitro* [15]. However, the Ab Hu-pyro-Glu showed no signals in the ECM of cultured NHEKs, whereas both the Ab to the NC16A domain (Ab Hu-NC16A) and the Ab HK139 were able to detect large amounts of COL17 as migration tracks (**Fig. 3A**). Consistent with the IIF findings, Western blotting using ECM samples of NHEKs showed that Ab Hu-pyro-Glu was unable to detect the 120-kD ectodomain of COL17, whereas both the Ab Hu-NC16A and the Ab HK139 were able to detect that ectodomain (**Fig. 3B**). These results suggest that migrating NHEKs cleave COL17 at sites different from those of the normal steady-state *in vitro*. To address this further in an *in vivo* setting, we tested the reactivity of the Abs on post-operated, ulcerated wound skin as described in the Material and Methods. It was found that physiological N-terminal cleavage at Gln^{525}, detectable by the Ab Hu-pyro-Glu, was diminished at the DEJ of the leading-edge epidermis, whereas slightly

reduced but distinct expression of COL17 was detectable by the Ab Hu-NC16A (**Fig. 3C, Supplement Fig.1A, B**). These results suggest that ectodomain shedding of COL17 is differentially regulated also in *in vivo* human wound skin. In addition, different expression pattern was observed in a region intermediate between the re-epithelializing edge and normal skin among different samples. The expression of the Ab Hu-pyro-Glu (region 2 in each figure) was much weaker in Case 1 and 3 than in Case 2 (**Fig. 3C and Supplement Fig1A**). The differences may reflect the difference in time elapsed after operation (25, 35 and 37 days, respectively).

Passive transfer of Abs to neoepitopes on candidate physiological cleavage sites of mouse COL17 ectodomain fails to reproduce blistering disease in mice

The final question was whether Abs to neoepitopes on the cleavage sites of the shed ectodomain of COL17 are pathogenic for blister formation. Because the cleavage-site-specific Ab Hu-pyro-Glu did not react with the COL17-humanized mice skin (not shown), we tried to generate another Ab targeting a candidate physiological cleavage site of mouse COL17. Based on the facts that Gln^{525} of human COL17 is highly conserved among different species (**Fig. 1B**), we hypothesized that the amino acid Gln^{532} of mouse COL17, corresponding to the Gln^{525} of human COL17, may be a major cleavage site of *in vivo* mouse skin. In addition, we speculated that the N-terminal Gln^{532} may be modified by changing into pyro-Glu as is seen for the human N-terminal Gln^{525} of COL17. To address these hypotheses, we produced another rabbit Ab, Mo-pyro-Glu, which targets

the candidate N-terminal cleavage site of mouse COL17, of which Gln^{532} has been changed into pyro-Glu (**Fig. 1B**). The Ab Mo-pyro-Glu strongly reacted with the DEJ of the WT mouse skin, whereas it failed to react with the COL17-lacking mouse (*Col17*$^{-/-}$) skin (**Fig. 4A**), supporting the idea of specificity of the Ab to mouse COL17. In addition, similar to the Ab Hu-pyro-Glu, the Ab Mo-pyro-Glu reacted with the epidermal side of artificial blisters of NMS pre-incubated with 1-M NaCl for 14 hrs (**Fig. 4A**), although its reactivity was lost on skin that had been incubated with 1-M NaCl for 24 hrs as observed by Ab Hu-pyro-Glu (not shown). The Ab Mo-pyro-Glu immunoprecipitates shed mouse COL17 ectodomain (**Fig.4B**), and Western blotting showed that the Ab Mo-pyro-Glu specifically reacted with the shed ectodomain of mouse COL17 but not with the full-length form (**Fig. 4C, D, E**). Western blotting using the highly concentrated Ab Mo-pyro-Glu and cell lysate from NMKs had the result that the Ab was able to react weakly with the 180-kD full-length mouse COL17 (not shown).

To address the pathogenic roles of Abs targeting the neoepitopes on the shed COL17 ectodomain in blister formation, we performed passive transfer of the Ab Mo-pyro-Glu and the Ab Mo-NC14A into neonatal and 8-wk-old C57BL/6J mice. Affinity-purified Ab Mo-pyro-Glu (10.72 μg/μl) with immunized peptides strongly reacted with the DEJ of the NMS, as observed by IIF (**Fig. 4A**) at more than 1:32,000 dilution (not shown). Although high concentrated Ab Mo-pyro-Glu was able to react weakly with the 180-kD full-length mouse COL17 , we used the Abs for passive-transfer experiments without immunoadsorption, since the treatment did not solely change the reactivity of the Ab Hu-pyro-Glu to the NHS, as

mentioned above. As a result, high-dose passive transfer of the affinity-purified Ab Mo-pyro-Glu failed to induce skin fragility in any of the recipient mice by 48 hrs after injection (n=6, **Fig. 4F**), although DIF studies revealed strong deposition of rabbit IgG and mouse C3 at the DEJ in all recipient mice (**Fig. 4G**). In contrast, 3 out of the 4 neonatal mice that had received the Ab Mo-NC14A, which was affinity-purified by protein A (84.29 μg/μl) and which reacts with NMS at more than 1:12,800 dilution, showed epidermal detachment by gentle mechanical frictions (**Fig. 4F**). Interestingly, DIF studies from the diseased mice revealed *in vivo* deposits of rabbit IgG at the DEJ, although complement activation was very faint or largely absent (**Fig. 4H**).

For adult mice, we performed the passive transfer of antiserum containing the Ab Mo-pyro-Glu which strongly reacts with the DEJ of NMS as observed by IIF at over 1:3,200 dilution (not shown). Repeated passive transfer of antiserum containing the Ab Mo-pyro-Glu, totaling 2,500 μl per mouse, did not induce any skin disease in recipient WT adult mice (n=4), although the *in vivo* deposition of rabbit IgG and mouse C3 was observed at the DEJ of the skin in all the recipient mice (**Fig. 4G**). In addition, the treated mice had circulating Abs that reacted with the DEJ of the NMS at more than 400x dilution (**Fig. 4F**). Control mice that received the same dose of pre-immuno rabbit serum showed no phonotypical abnormalities, and no rabbit IgG or mouse C3 deposition was observed in the skin (not shown).

Discussion

COL17 is a key molecule for maintaining stable adhesion at the DEJ. Innate and acquired dysfunctions of the molecule respectively leads to the blistering disorders of non-Herlitz junctional epidermolysis bullosa [10] and pemphigoid diseases [3]. These blistering diseases suggest that COL17 plays a vital role in the stability of the skin. In contrast, here we showed certain amounts of the COL17 ectodomain are physiologically cleaved even in steady-state normal human and mouse skin. COL17 is known to be expressed not only at the DEJ but also basolaterally and in the cytoplasm [11, 15]. However, the cleaved COL17 ectodomain detected by Abs targeting cleavage sites of the molecule (Ab Hu-pyro-Glu) was exclusively observed along the DEJ. This finding suggests that ectodomain shedding occurs only beneath the basal keratinocytes, probably within the hemidesmosomes. Similar findings have been reported for mAb 1337, another ectodomain-specific mAb reacting with bovine COL17 [11].

Interestingly, we found that majority N-terminal Gln^{525} of the shed COL17 ectodomain in culture medium is not cyclized into pyro-Glu, which can be spontaneously or enzymatically cyclized. Although it is still uncertain whether N-terminal cyclization of shed COL17 ectodomain is mediated by which pathways; Ab Hu-pyro-Glu identified cleaved COL17 ectodomain with cyclized N-terminus *in vivo*. Notably, that the cleaved COL17 ectodomain with cyclized N-terminus was diminished leading edge epidermis in the wound skin.

It is well characterized that migrating activated keratinocytes express distinct

adhesion molecules, such as integrin α3β1 and α2β1, at the BMZ *in vitro* [26, 27]. In addition, wound keratinocytes are known to express these distinctive integrins [28]. These findings suggest that different molecules are involved in keratinocyte migration. Previously, we showed that migrating keratinocytes cleave and leave the ectodomain of COL17 in the ECM as seen by the migration track *in vitro*, which was revealed by Ab HK139, which targets the N-terminal cleavage site at Leu^{524} [15]. This observation was rather associated with pathological settings, because we observed actively migrating keratinocytes. In contrast, here we showed that the Ab Hu-pyro-Glu did not react with the migration tracks of cultured NHEKs. Therefore, it is likely that the N-terminal cleavage of COL17 at Gln^{525} is associated with a physiological—and not a pathological—setting, suggesting that cleavage of COL17 is tightly regulated depending on the settings. However, what is the physiological function of COL17 ectodomain shedding in normal skin? It has been postulated that COL17 ectodomain shedding plays physiological roles associated with the migration, proliferation and differentiation of basal keratinocytes [13]. However, there has been no direct evidence for this. One possible physiological role may relate to the differentiation of basal keratinocytes, because they must detach from the ECM in order to differentiate into the spinous and cornified layers. Alternatively, normal epidermis may be a more dynamic organ that actively communicates with ECM proteins through cell-surface receptors including COL17, whereby ectodomain shedding of the molecule is essential even a normal setting.

The present study also suggests the presence of binding proteins of cleaved

COL17 ectodomain in the ECM, because IIF studies of cleavage-site-specific Abs on 1-M NaCl-split skin showed that the cleaved COL17 ectodomain remains on the epidermal side of artificial blisters. We and another group have reported that the C-terminus of COL17 binds with laminin 332 *in vitro* [23, 29]. Because laminin 332 is always present on the dermal side of 1-M NaCl-split skin [3, 30], the present data indicate that another binding molecule, or molecules, on the epidermal side is present in the ECM, which binds with the shed COL17 ectodomain more strongly than laminin 332 does. A candidate molecule is integrin α6, which is known to be able to bind with the ectodomain of COL17 [31, 32]. Alternatively, additional cleavage may occur in the C-terminus of COL17 when artificial blisters form by 1M NaCl. In any case, the present data suggest that the shed ectodomain of COL17 binds with another molecule, or molecules, at the DEJ.

The ectodomain shedding of COL17 can evoke neoepitopes on the shed forms, which can be targeted by autoAbs of pemphigoid patients [16, 17]. Previously, we showed that linear B-cell epitopes have significantly increased antigenicity around cleavage sites within the NC16A domain, regardless of the physiological cleavage sites predicted *in silico* [15]. However, the detailed pathomechanism of neoepitope generation has not been elucidated, and pathological role of autoAbs to neoepitopes is still uncertain *in vivo*. This study revealed that modified N-terminal cleavage sites whose Gln has been changed into pyro-Glu produce distinct neoepitopes at the N-terminal cleavage sites. We previously generated transgenic COL17-humanized mice in which mouse COL17 was replaced by human COL17. The mice enabled us to reproduce blistering disease by passively

transferring Abs to human COL17 [9, 21]. Although the COL17-humanized mouse skin expresses full-length human COL17, the Ab Hu-pyro-Glu did not stain at the DEJ. Similarly, the cleavage-site-specific Ab HK139 targeting N-terminal Leu^{524} did not react with the COL17-humanized mouse skin (not shown, unpublished data). It is still uncertain why these Abs lost the reactivity to the human COL17 in the transgenic mice. One possible mechanism is that the cleavage of human COL17 in the COL17-humanized transgenic mouse skin may be different from it occurs in NHS. However, further studies are needed to elucidate in detail. In this study, we produced rabbit Abs targeting a candidate cleavage site of mouse COL17 ectodomain. Passive transfer of Abs targeting the candidate pyro-Glu N-terminus of mouse COL17 ectodomain failed to induce blistering diseases in neonatal and adult WT mice, even though injected rabbit IgG bound with mouse COL17 and activated mouse C3 *in vivo*. Previous studies have shown that *in vivo* deposition of Abs to the NC16A domain (the NC14A domain for mouse COL17) of COL17 associated with complement activation is a hallmark of neonatal BP models [8, 33], although we recently found that complement activation is not necessary for blistering diseases in mice [21, 34]. In any case, the present study found no evidence that Abs targeting a candidate physiological N-terminal cleavage sites are pathogenic *in vivo*. However, epitopes can be significantly changed in regions far from the cleavage sites. For example, the mAb NC16A-3, whose epitope is at least 19 aa C-terminal from the physiological cleavage site within the NC16A domain of COL17, preferentially reacts with the shed ectodomain but not with the full-length form [20]. In addition, the 15^{th} collagenous domain is known to be a candidate epitope for autoAbs from LAD patients [35],

which suggests that the conformation of the COL17 ectodomain may change drastically after cleavage within the NC16A domain. Therefore, the current study does not rule out the possibility that Abs reacting with other neoepitopes far from the physiological cleavage sites may be pathogenic for blister formation.

In summary, this study showed that the ectodomain of COL17 is physiologically cleaved *in vivo* in human skin as well as mouse skin, which produces distinct neoepitopes at the cleavage sites with pyroglutamate N-termini. Although rabbit Abs to the neoepitopes bind with mouse COL17 and activate murine complements *in vivo*, blistering disease is not introduced, suggesting that immune reaction to the neoepitopes on the physiological N-terminal cleavage sites will not be pathogenic.

Acknowledgements

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Figure Legends

Figure. 1

The physiological N-terminal cleavage site Gln^{525} of human COL17 is highly conserved among different species. **(A)** Physiologically, the ectodomain of human COL17 cleaves at different sites within the NC16A domain, including at

Asp^{514}, Leu^{524}, Gln^{525} and Gly^{526} (red arrowheads). Epitopes of Abs HK139 and Hu-pyro-Glu are shown. The N-terminal Gln of the immunized peptides for the Ab Hu-pyro-Glu has been chemically changed into pyro-Glu (asterisk). TM: transmembrane. **(B)** The N-terminal cleavage site Gln^{525} for human COL17 (arrow, red color) is highly conserved among different species, suggesting that Gln^{525} is a vital amino acid. The corresponding epitope of immunized peptides to produce the Ab Mo-pyro-Glu is illustrated. The N-terminal Gln of the immunized peptides for the Ab has also been chemically changed into pyro-Gln (asterisk).

Figure. 2

Ectodomain shedding of human COL17 in *in vivo* human skin. **(A)** Western blotting using cell lysate (L) and culture medium protein (M) from NHEKs shows that the Ab Hu-pyro-Glu weakly reacts only with the 120-kD shed ectodomain (arrow) and not with the 180-kD full-length form of COL17. In contrast, Abs 09040 and Hu-NC16A, which respectively target the C-terminus and the NC16A domain, react with both the full-length and the shed forms. Ab Hu-NC16A preferentially react with 120-kD shed ectodomain than full-length form. **(B)** IP using Abs Hu-NC16A, Hu-pyro-Glu before and after the immunoadsorption using synthesized R7 peptide spanning Asp^{522} to Gln^{545}. IP samples from cell lysate (upper lane) and culture medium (lower lane) of NHEKs were detected by Ab 09040 and mAb NC16A-3 respectively. Ab Hu-pyro-Glu before immune adsorption weakly reacts with full-length COL17, whose reactivity was eliminated after immunoadsorption of the Abs. However, reactivity of Ab Hu-pyro-Glu to shed ectodomain was diminished after the immunoadsorption. **(C)**

Immunoreactivity of the Ab Hu-pyro-Glu on NHS did not differ before versus after the immunoadsorption (concentration of Abs: 0.005μg each). Scale bar: 50 μm. **(D)** Treatment of NHEKs culture medium with recombinant human glutaminyl-peptide cyclotransferase. Western blotting shows 120kD COL17 ectodomain detected by Ab Hu-pyro-Glu increased in medium protein treated with glutaminyl-peptide cyclotransferase, but reactivity by mAb NC16A-3 isn't changed. The Ab Hu-pyro-Glu can immunoprecipitate small amounts of COL17 ectodomain from non-treated culture medium (3ml); in contrast, the Ab immunoprecipitates increased 120kD COL17 ectodomain from the glutaminyl-peptide cyclotransferase-treated medium protein, which was detected by mAb NC16A-3. **(E)** Spontaneous cyclization of N-terminal Gln^{525} of shed COL17 ectodomain into pyro-Glu. Long time incubation of NHEKs culture medium at 37°C produced increased amounts of COL17 ectodomain which is detectable by immune adsorbed Ab Hu-pyro-Glu of COL17 as time-dependent manner (upper lane). In contrast, reactivity of the 120-kD Ab NC16A-3 decreased, indicating certain amounts of COL17 ectodomain is degraded during the incubation (lower lane, stripped membrane was used). (**F**) The Ab Hu-pyro-Glu fails to react with skin sections obtained from a COL17-lacking epidermolysis bullosa patient. The Ab reacts with the epidermal side of artificial blisters of NHS induced by pre-incubation with 1-M NaCl for 14 hrs, suggesting the presence of an unknown binding molecule(s) that anchors the shed COL17 ectodomain in the ECM. Stars: blisters. Scale bar: 50 μm.

Figure. 3

Ectodomain shedding of COL17 in migrating keratinocytes. **(A)** The cleaved ectodomain of COL17 in the ECM (arrows in left panel, green in right panel) of the NHEKs is detectable by both the Abs targeting the NC16A domain (Ab Hu-NC16A) and the cleavage site of Leu^{524} (Ab HK139), whereas the Ab Hu-pyro-Glu and normal rabbit IgG as control fails at such detection. Red: Rhodamine phalloidin. Scale bar: 25 μm. **(B)** Western blotting using ECM samples (E) and culture medium protein (M) of NHEKs shows that the Ab Hu-pyro-Glu is unable to detect the 120-kD cleaved ectodomain of COL17 in the ECM, whereas the Ab Hu-NC16A and the Ab HK139 is able to detect this ectodomain. **(C)** An example of post-operation ulcers from Case 1 (a 26 year-old man, 25 days after the operation). In contrast to the Abs Hu-NC16A and HK139, signals along the DEJ by the Ab Hu-pyro-Glu are diminished at the leading edge and re-epithelialized hypertrophic epidermis (regions 3 and 2 respectively, arrowheads). Note gradual reduction of Ab Hu-pyro-Glu staining along the DEJ in transitional region from normal to hypertrophic skin (region 1, arrows). The lowest picture is high magnification of Ab-Hu-pyro-Glu in region 1. Staining of Green: Abs. Red: PI.

Figure. 4

Pathogenic roles of Abs to neoepitopes on physiological cleavage sites of mouse COL17. **(A)** IIF using 2.25 ng/μl diluted affinity-purified Ab Mo-pyro-Glu shows strong reaction with the DEJ of the WT mouse skin, whereas it fails to react with COL17-lacking mouse (*Col17*$^{-/-}$) skin. The Ab reacts with the epidermal side of 1-M NaCl-induced artificial blisters of NMS. Stars: blisters. Scale bar: 50 μm. **(B)**

IP of shed COL17 ectodomain from NMKs culture medium by Mo-pyro-Glu. Ab Mo-pyro-Glu immunoprecipitates 120kD mouse COL17 ectodomain, which was detected by Ab Mo-NC14A. Input: concentrated medium protein. **(C)** Western blotting using cell lysate (L) and culture medium (M) of NMKs. In contrast to the Ab Mo-NC14A reacting with both the full-length form and the shed ectodomain of mouse COL17, the Ab Mo-pyro-Glu only reacts with the shed 120-kD ectodomain (arrow). The dim band around 150 kD is a non-specific signal. **(D)** The recombinant GST-Mo-NC14A protein is seen to migrate around 37 kD by Coomassie staining. Small fragments between 25 kD and 37 kD are expected to be degraded or truncated proteins, which can be cleaved off by PreScission protease (PP, star). **(E)** Western blotting using recombinant Mo-NC14A-His·HSV protein (Lane 1) and control protein from empty pET-44a vector (Lane 2). Similar to Anti-HSV-tag Abs, the Ab Mo-NC14A reacts with Mo-NC14A-His·HSV, which migrates around 80 kD. **(F)** A neonatal C57BL/6 WT mouse 48 hrs after receiving intraperitoneal injection of 250 μg/g body weight affinity-purified Ab Mo-pyro-Glu. No epidermal detachment is observed (arrows), but DIF studies demonstrate *in vivo* deposition of rabbit IgG and mouse C3 (arrowheads) at the DEJ of skin from the injected mice. In contrast, a neonatal C57BL/6 WT mouse that received intraperitoneal injection of 2,000 μg/g body weight purified whole IgG containing Ab Mo-NC14A shows skin fragility (black arrowheads) associated with *in vivo* deposition of rabbit IgG (white arrow heads), but mouse C3 is not activated. No distinct abnormal findings are observed in the control mice that received normal rabbit IgG (arrows). **(G)** An adult C57BL/6 mouse that has received subcutaneous injection of total 2,500 μl of anti-serum containing the Ab

Mo-pyro-Glu. No phenotypic abnormalities are observed. DIF studies demonstrate *in vivo* deposition of rabbit IgG (arrows) and mouse C3 (arrowheads) at the DEJ of the back skin of the injected mice. Scale bar: 100 μm. **(h)** IIF studies using sera from adult WT mice that were injected with the Ab Mo-pyro-Glu. The treated mice, but not the control mice, have circulating Abs that strongly react with the DEJ of NMS at 400x dilution. Scale bar: 100 μm.

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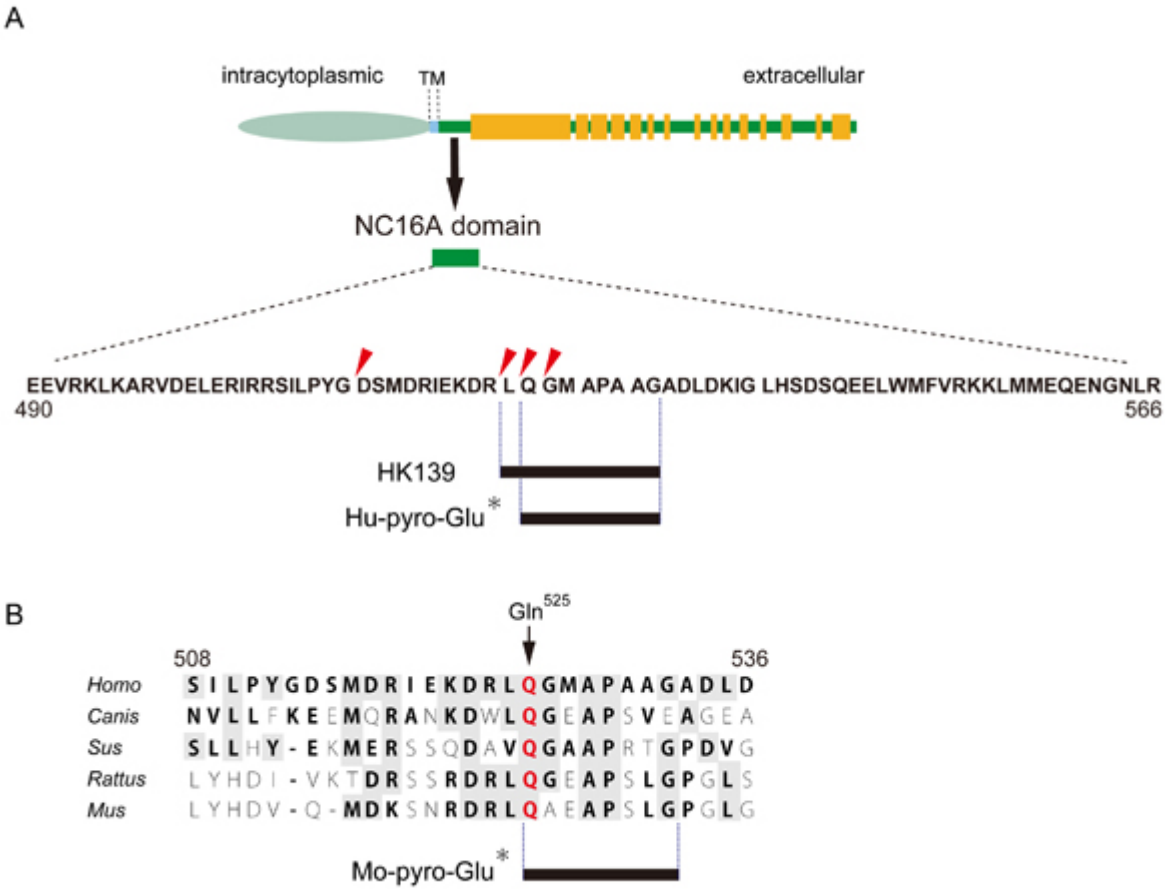
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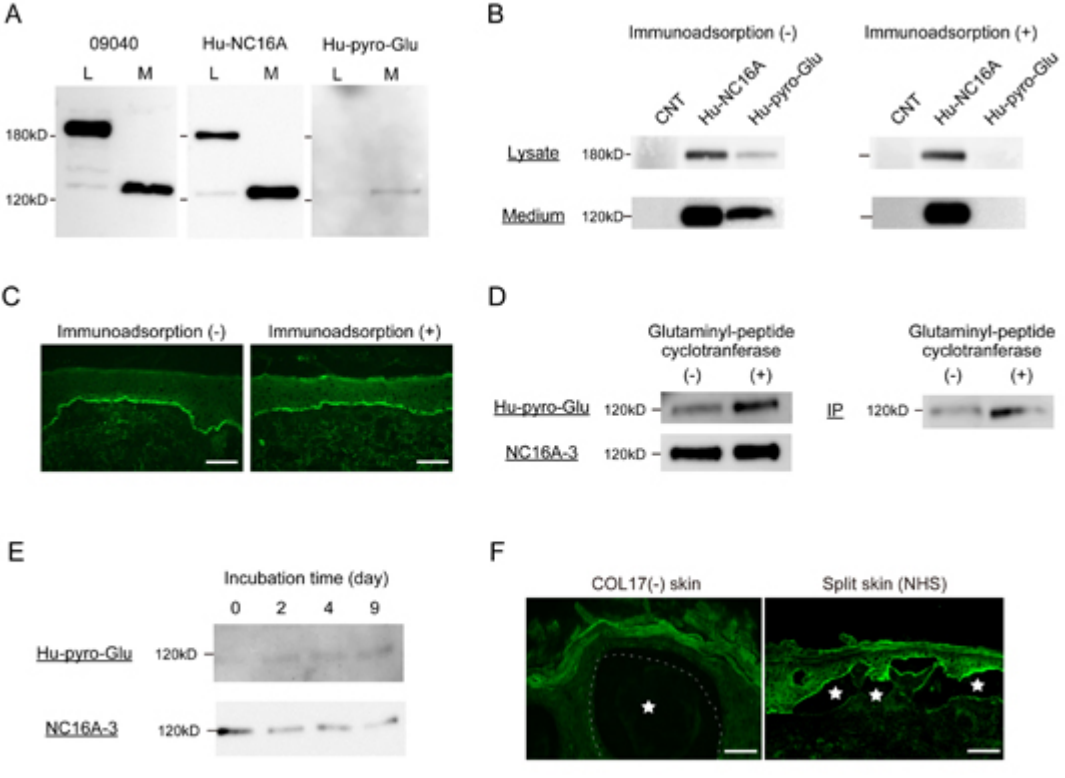
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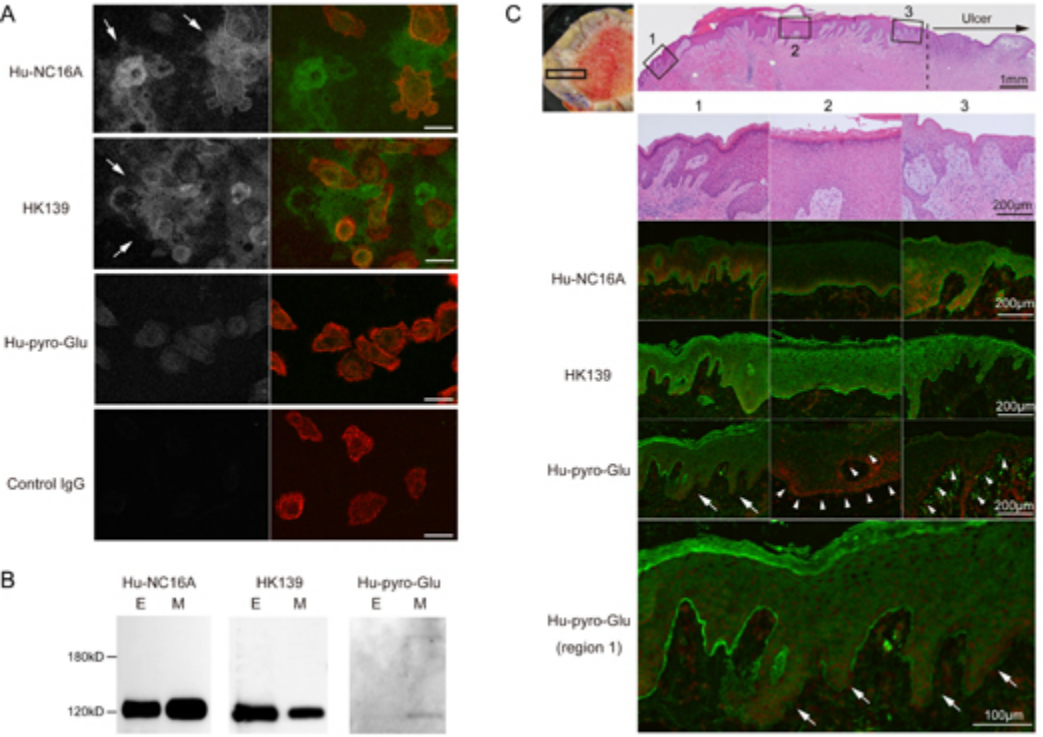
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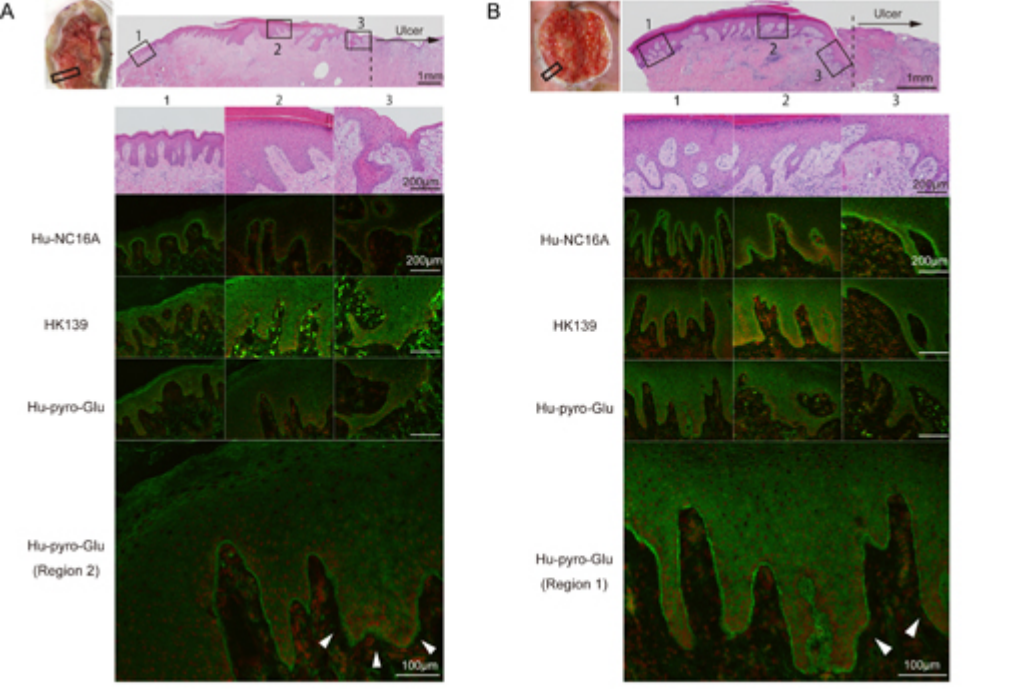
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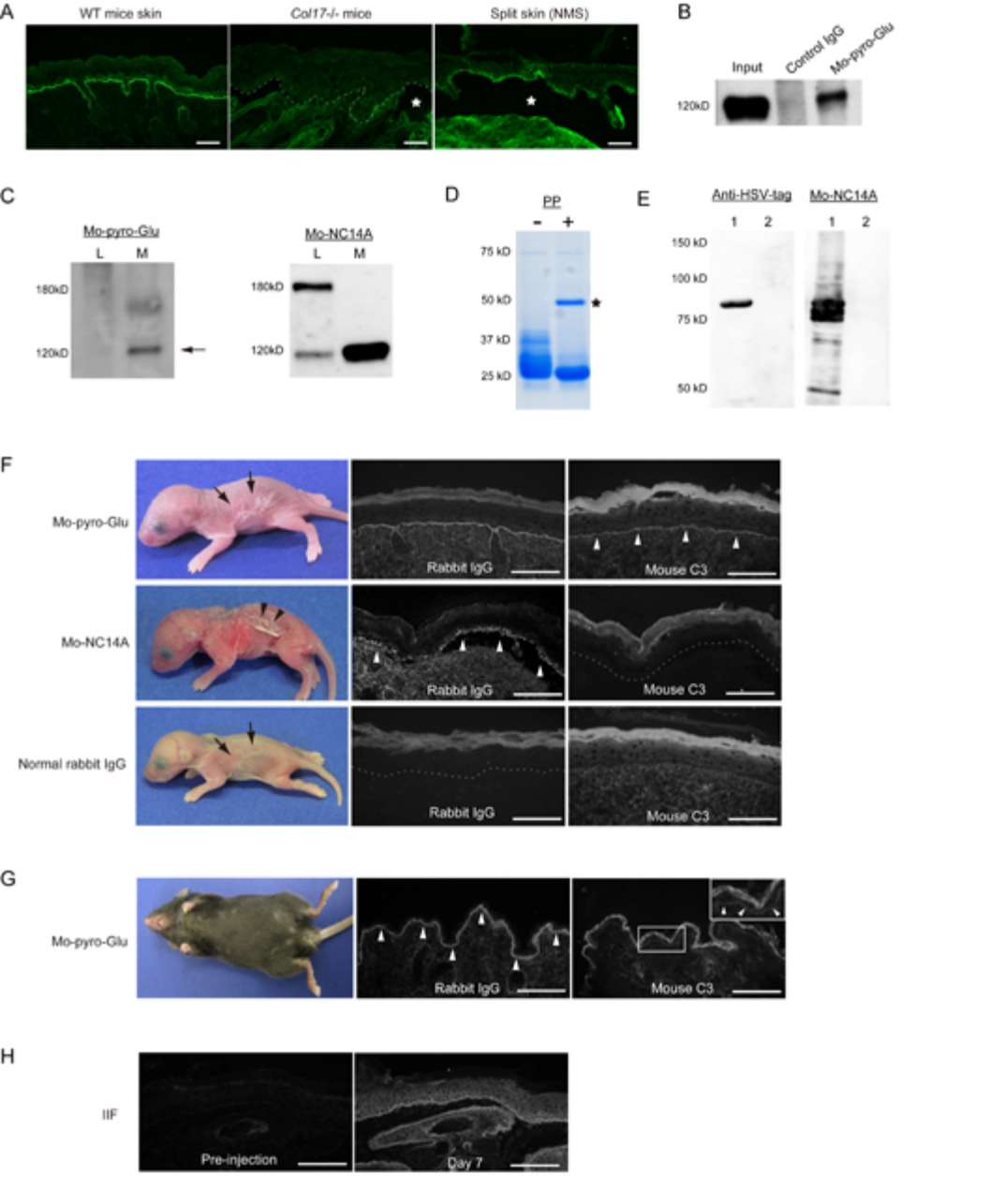
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PP
-
+
75 kD
50 kD
37 kD
25 kD
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Mo-NC14A
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2
1
2
150 kD
100 kD
75 kD
50 kD
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Mo-NC14A
Normal rabbit IgG
Rabbit IgG
Mouse C3
Rabbit IgG
Mouse C3
Rabbit IgG
Mouse C3
G
Mo-pyro-Glu
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H
IIF
Pre-injection
Day 7