Generation of a Functional Non-Shedding Collagen XVII Mouse Model: Relevance of Collagen XVII Shedding in Wound Healing

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Collagen XVII is a hemidesmosomal anchorage molecule of basal keratinocytes that promotes stable epidermal-dermal adhesion. One unique feature of collagen XVII is that its collagenous ectodomain is constitutively released from the cell surface by a disintegrin and metalloproteinases (ADAMs) through cleavage within its juxtamembranous linker domain. The responsivity of shedding to environmental stimuli and the high stability of the released ectodomain in several tissues suggests functions in cell detachment during epidermal morphogenesis, differentiation, and regeneration, but its physiologic relevance remained elusive. To investigate this, we generated knock-in mice, which express a functional non-sheddable collagen XVII mutant, with a 41 amino acid deletion in the linker domain spanning all ADAM cleavage sites. These mice showed no macroscopic phenotypic changes, were fertile, and had a normal lifespan. Prevention of collagen XVII shedding interfered neither with skin development nor with epidermal adhesion and differentiation. However, it led to faster wound closure due to accelerated re-epithelialization at the wound edges where shedding of wild-type collagen XVII was strongly induced. Taken together, we have successfully generated a functional non-shedding collagen XVII mouse model, which represents a powerful tool to investigate the pathophysiologic relevance of ectodomain shedding during wound regeneration and cancer invasion.

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INTRODUCTION

Collagen XVII, also known as 180-kDa bullous pemphigoid autoantigen (BP180), is a hemidesmosomal anchorage molecule of basal keratinocytes that mediates the epidermal adhesion to the underlying basement membrane (BM). This type II oriented transmembrane protein is an important contributor for the maintenance of type I hemidesmosomes (HDs) in the skin. Abrogation of collagen XVII expression in humans and mice results in immature HDs, weakened dermal-epidermal adhesion, and skin blistering along the BM, documenting its relevance for dermal-epidermal adhesion (Franzke et al., 2005; Nishie et al., 2007, 2011; Tasanen et al., 2004; Van den Bergh et al., 2010). Anchorage through collagen XVII has also been implicated in the maintenance of the self-renewal of hair follicle stem cells (Tanimura et al., 2011), which is documented by hair loss due to collagen XVII deficiency (Franzke et al., 2005; Nishie et al., 2007; Hurskainen et al., 2012).

We have previously shown that the ectodomain of collagen XVII is constitutively shed from the cell surface by a disintegrin and metalloproteinase (ADAM) members (Franzke et al., 2002, 2009). The cleavage of collagen XVII within its juxtamembranous linker domain shows no sequence but structural specificity (Franzke et al., 2004) and mainly depends on ADAMs 9 and 10 (Franzke et al., 2009). In vitro studies have demonstrated that cell surface proteolysis of collagen XVII is responsive to cytokines or reactive oxygen species and can be modulated by cell membrane microlocalization or environmental interactions with its extracellular binding ligands laminin-332 and collagen IV (Franzke et al., 2002, 2009; Nishie et al., 2011; Zimina et al., 2005). This kind of responsivity associates collagen XVII shedding with modulation of keratinocyte detachment during epidermal homeostasis and regeneration. In addition, the high stability of the released ectodomain in different tissues, including skin and mucosa, suggests that it has alternative functions (Franzke et al., 2005; Hofmann et al., 2009; Huilaja et al., 2008). However, the biological and pathologic functions of collagen XVII ectodomain shedding remained elusive over the years, especially due to the lack of suitable non-shedding in vivo models.

Previous attempts to elucidate the physiologic consequences of the absence of collagen XVII cell surface proteolysis using ADAM-deficient mouse systems failed due to cleavage redundancy among constitutive ADAMs toward collagen XVII (Franzke et al., 2002, 2009) and the severe phenotypes caused by the lack of shedding of membrane proteins other than collagen XVII, including EGFR-ligands

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Abbreviations: ADAM, a disintegrin and metalloproteinase; BM, basement membrane; HD, hemidesmosome

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and Notch (Franzke et al., 2012; Weber et al., 2011). Furthermore, it has recently been demonstrated that deletion of the complete linker domain is not sufficient to prevent collagen XVII shedding in vivo (Hurskainen et al., 2015).

Here we present a successful strategy to create functional collagen XVII non-shedding mice. The generation of this knock-in mouse model was based on the in vitro identification of a 41 amino acid deletion within the juxtamembranous linker domain of collagen XVII that prevented shedding, while it preserved triple helix formation and cell surface targeting. Exclusive expression of non-sheddable collagen XVII had no effect on epidermal architecture and differentiation in intact resting skin but led to accelerated re-epithelialization at the edges of cutaneous wounds, in which the shedding of wild-type collagen XVII was strongly induced. Thus, our non-shedding mice represent an excellent model to investigate the in vivo significance of collagen XVII ectodomain shedding in invasive processes during wound healing or carcinogenesis.

RESULTS

Identification and characterization of a functional non-sheddable collagen XVII mutant

To investigate the physiologic functions of ectodomain shedding of collagen XVII, we aimed to generate mice that express a functional non-sheddable collagen XVII molecule. Although sequence alignment revealed about 86% amino acid identity between the human and murine collagen XVII molecules, the sequences of their juxtamembranous linker domains (human NC16A and murine NC14A) showed pronounced diversity with about 57% identity (see Supplementary Figure S1a online). Secondary structure prediction revealed conserved N- and C-terminal alpha-helical stretches in both sequences but a highly diverse middle part with reduced alpha-helices and an additional beta-sheet in the murine linker domain (Supplementary Figure S1b), which suggests different ADAM cleavage sites in human and murine collagen XVII. Therefore, we detected the N-termini of affinity-purified shed murine collagen XVII ectodomain by multiple liquid chromatography tandem mass spectrometry analysis (Figure 1a–c). This analysis resulted in the identification of Asn^{527}, Leu^{531}, and Gln^{532} as three distinct N-termini within the murine NC14A domain (Figure 1d). Surprisingly, the clustered cleavage sites in murine collagen XVII showed very similar localization to the previously identified sites in human collagen XVII (Nishie et al., 2010) with nearly identical distances to the cell membrane (Supplementary Figure S1c), demonstrating their very similar structural accessibility to proteolytical cleavage by ADAMs. This is in line with the fact that similar ADAMs shed collagen XVII in human and murine keratinocytes (Franzke et al., 2002, 2009).

Based on the cleavage sites in murine collagen XVII and our previously successful strategy to prevent shedding of human collagen XVII (Franzke et al., 2004), we generated five deletion mutants by site-directed mutagenesis (Qi & Scholthof, 2008) to find the shortest deletion within the NC14A domain in collagen XVII which prevent shedding while preserving molecule functionality (Figure 1e). The analysis of ectodomain release of these mutants revealed the amino acid stretch from Glu^{510} to Tyr^{550} (Δ41) as shortest deletion, which completely prevented the ADAMs mediated cleavage (Figure 1e).

The functionality of the Δ41 collagen XVII mutant was approved by insensitivity to endoglycosidase H digestion as prove for its normal *trans*-Golgi passage (see Supplementary Figure S2a online), good accessibility for cell surface biotinylation that demonstrates its proper cell surface integration (Supplementary Figure S2b), and normal collagen triple helix formation as evidenced by similar resistance to limited trypsin digestion at temperatures of around 43°C (Supplementary Figure S2c). Further the collagenous nature of the Δ41 mutant was proven by specific digestion with highly purified collagenase form III from *Clostridium histolyticum* that selectively target triple-helical collagenous sequences (data not shown).

Generation of collagen XVII non-shedding knock-in mice

The collagen XVII non-shedding mice, which exclusively produce mutant Δ41 collagen XVII (Supplementary Figure S2d), were generated using the knock-in strategy of introducing a 123 bp deletion into exon 18 of the *Col17a1* gene. The $Col17a1^{\Delta NS}$ targeting vector was transfected into the C57BL/6N ES cell line Tac and the puromycin resistance cassette was excised (Figure 2a). These ES cells were used to generate founder mice of the non-shedding collagen XVII mice, further referred as $Col17a1^{\Delta NS/\Delta NS}$ mice, which were maintained in a C57BL/6 background. Genotyping of the offsprings of intercrossed $Col17a1^{\Delta NS/+}$ animals by PCR identified $Col17a1^{\Delta NS/\Delta NS}$, $Col17a1^{\Delta NS/+}$, and $Col17a1^{+/+}$ littermates (Figure 2b), which were distributed in an expected Mendelian ratio, indicating no increased lethality of $Col17a1^{\Delta NS/\Delta NS}$ embryos. Immunoblot analysis of $Col17a1^{\Delta NS/\Delta NS}$ skin and keratinocytes demonstrated complete loss of the 120-kDa ectodomain, confirming non-shedding of Δ41 collagen XVII in vivo and in vitro (Figure 2c and d).

$Col17a1^{\Delta NS/\Delta NS}$ and $Col17a1^{\Delta NS/+}$ mice were indistinguishable from their $Col17a1^{+/+}$ littermates (Figure 3a), bred normally, showed no differences in their body mass (see Supplementary Figure S3a online), and had a normal lifespan. These mice developed neither skin blisters on the paws and around the genitals nor loss of hair or nails (Supplementary Figure S3b and c), which were caused by knockout of collagen XVII in mice (Hurskainen et al., 2012; Nishie et al., 2007).

Collagen XVII ectodomain shedding is dispensable for epidermal architecture and differentiation

Histologic analysis revealed no obvious changes in skin architecture in $Col17a1^{\Delta NS/\Delta NS}$ mice (Figure 3b). To assess whether the exclusive expression of non-shed collagen XVII in $Col17a1^{\Delta NS/\Delta NS}$ mice affected HD functionality and skin integrity, back skin of mice of different ages (from birth to postnatal day 60, P60) was examined for distribution and expression of several BM and HD components. No differences were seen for non-shedding collagen XVII (Figure 3c), for its binding partners $\alpha_6\beta_4$ integrin and laminin-332 (Figure 3d, see Supplementary Figure S4a online), and for β1 integrin or collagen VII (data not shown) in $Col17a1^{\Delta NS/\Delta NS}$ skin compared with $Col17a1^{+/+}$ skin, suggesting that the composition of the BM zone in $Col17a1^{\Delta NS/\Delta NS}$ mice was not grossly altered. Furthermore, analysis of the ultrastructural localization of Δ41 collagen XVII in the skin revealed similar distribution and frequency in