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Long-Term Faithful Recapitulation of Transglutaminase 1–Deficient Lamellar Ichthyosis in a Skin-Humanized Mouse Model, and Insights from Proteomic Studies

Transglutaminase 1 (TG1)–deficient lamellar ichthyosis (LI) is associated with increased mortality in the neonatal period and has a marked impact on quality of life. No efficient treatment is available; current therapy only relieves some symptoms (Oji and Traupe, 2006). For the development of new therapeutic approaches and to further investigate molecular mechanisms underlying the pathophysiology of LI, a stable, long-lived preclinical model is needed, which fully recapitulates the human skin phenotype.

In vivo studies in human skin are limited by ethical and practical considerations. The *Tgm1*$^{-/-}$ mouse does not recapitulate the human skin phenotype. Moreover, the mice die within the first hours of life (Matsuki *et al.*, 1998) because of impaired barrier function, underscoring the importance of TG1 for barrier formation (Candi *et al.*, 2005). Only transplanted *Tgm1*$^{-/-}$ mouse skin resembled the skin seen in severe ichthyosis (Kuramoto *et al.*, 2002). Choate and colleagues showed the feasibility of *ex vivo* and *in vivo* gene transfer for LI (Choate *et al.*, 1996; Choate and Khavari, 1997), although their model system only allowed short-term human skin regeneration. Moreover, a model of rat skin with TG1 deficiency was described (O'Shaughnessy *et al.*, 2010).

Using optimized tissue engineering and surgical conditions enabling stable human skin engraftment in athymic nude mice (García *et al.*, 2011), we have been able to develop a robust skin-humanized mouse model for TG1-deficient LI, involving persistent engraftment of bioengineered human skin, suitable for long-term, preclinical studies and for the investigation of molecular mechanisms.

We analyzed two LI patients and identified the compound heterozygous *TGM1* mutations c.377G>A (p.Arg126His) and c.876+2T>C (p.Glu253Valfs*2; patient 1) and c.428G>A (p.Arg143His) and c.877-2A>G (p.Phe293Serfs*38 or p.Phe293Valfs*2; patient 2), which were previously described (Farasat *et al.*, 2009). Punch biopsies obtained from these patients were used to isolate keratinocytes and fibroblasts after obtaining written, informed consent of the probands and institutional approval in accordance with the Declaration of Helsinki Principles. At 4–6 weeks after grafting of bioengineered skin equivalents, regenerated human skin grafts became visible and persisted in the recipient animals longer than 20 weeks, indicating stable engraftment of epidermal stem cells (data not shown).

Macroscopically, epidermal hyperplasia and an increased scaly hyperkeratosis completely matched the human skin phenotype. Using a human-specific antibody against involucrin, we could confirm the human origin of the grafts and clearly delineate the border between mouse and human skin. Light microscopy displays a very thick and packed stratum corneum (SC) in the LI grafts (Figure 1a).

TG1 activity and protein were absent in LI skin/grafts. Ultrastructural analysis revealed cholesterol clefts in the SC of the LI skin/grafts, which are important diagnostic markers typical for TG1 deficiency (Pigg *et al.*, 1998). In contrast, skin/grafts derived from healthy individuals were completely normal (Figure 1b).

The presence and spatial distribution of TG1 substrates, differentiation markers, and dermoepidermal junction (DEJ) constituents were assessed by immunostaining. The comparison between normal graft/skin and LI graft/skin showed that normal grafts mirrored normal skin and, similarly, alterations of epidermal differentiation in LI grafts matched those found in LI skin. The diffuse and upwardly shifted distribution of loricrin and involucrin indicates insufficient cross-linking of these proteins into the cornified envelope (CE). Filaggrin was expressed in the periphery of cells from the upper spinous layer in all samples. The early differentiation marker keratin 10, localized in viable suprabasal cell layers, remained unchanged (Figure 1c). Integrin-α6 decorates the DEJ in a continuous, linear manner. Collagen VII was present along the DEJ, indicating its correct formation in the grafts (Figure 1d).

To further explore changes in differentiation and to relate them to the previous results, we present, to our knowledge previously unreported, data on the analysis of the proteome of LI epidermis. Comparison between normal and LI skin/grafts provides valuable insights into molecular mechanisms involved in LI pathophysiology. Analysis of the skin-humanized mouse model yields very similar data to those observed in LI skin. Altogether, 147 proteins were identified (Supplementary Table S1 online). We focused on TG1 substrates and some conspicuous proteins, which may

Abbreviations: CE, cornified envelope; DEJ, dermoepidermal junction; KPRP, keratinocyte proline-rich protein; LI, lamellar ichthyosis; NMF, natural moisturizing factor; TEWL, transepidermal water loss; TG1, transglutaminase 1

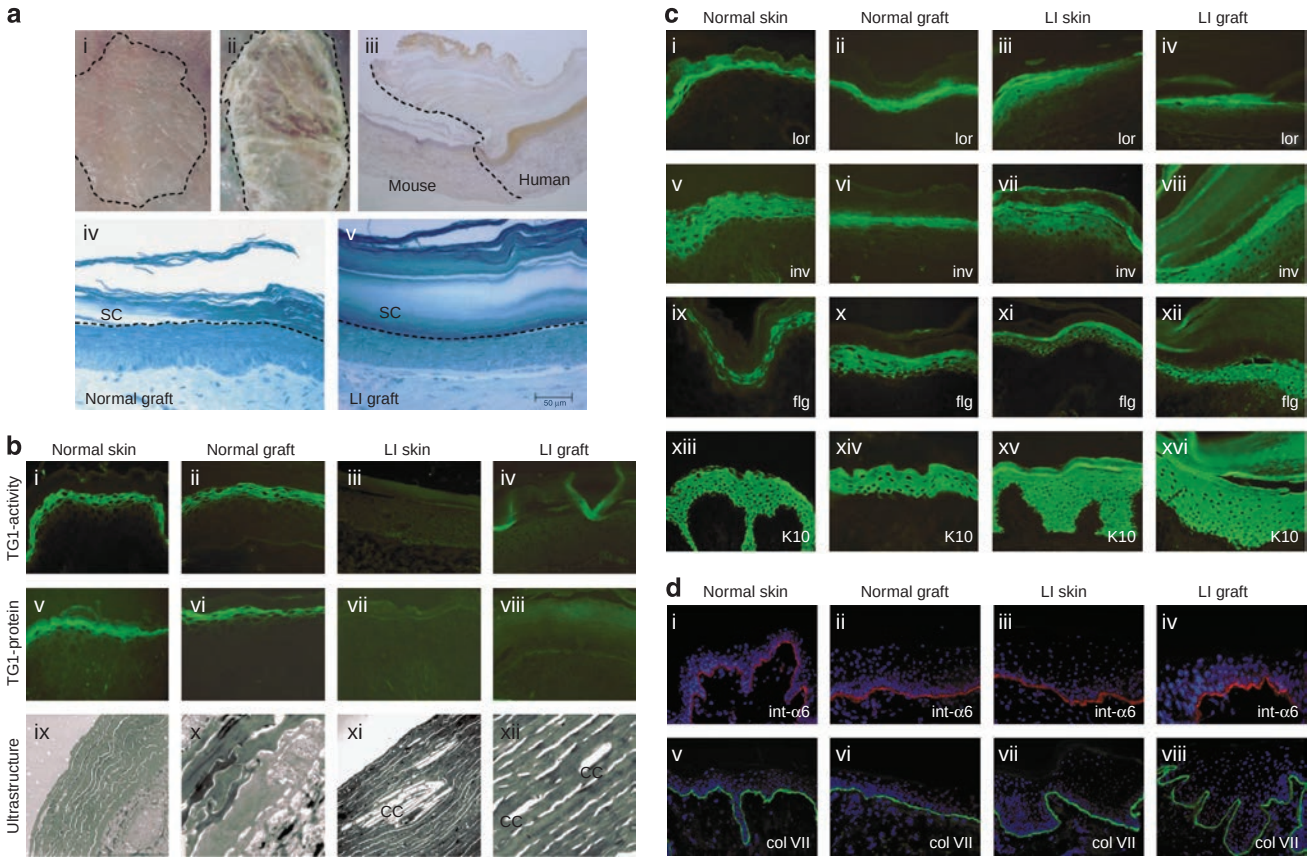

Figure 1. Characterization of the skin-humanized mouse model for transglutaminase 1–deficient lamellar ichthyosis. (**a**) Keratinocytes were seeded onto a fibroblast-populated fibrin-based matrix. Skin equivalents were grafted orthotopically onto the back of athymic nude mice. (i) Normal human regenerated skin and (ii) regenerated lamellar ichthyosis (LI) skin (patient 1) 12 weeks after grafting; (iii) peroxidase staining of involucrin (human specific) confirms the human skin phenotype; (iv) semithin sections, methylene blue staining, and morphology of normal and (v) LI grafts showing a very thick and packed stratum corneum (SC). Bar: iv, v = 50 µm. (**b** i–iv) Transglutaminase 1 (TG1) activity, (v–viii) TG1 protein, and (ix–xii) ultrastructure. Normal skin/grafts show the typical pericellular distribution of TG1 activity/protein in the stratum granulosum. Ultrastructurally, no cholesterol clefts are visible. In contrast, LI skin and LI grafts lacked TG1 activity/protein but displayed cholesterol clefts as typical ultrastructural markers. (**c** i–iv) Characterization of human and regenerated skin by immunostaining. TG1 substrates such as loricrin (lor), (v–viii) involucrin (inv), and (ix–xii) filaggrin (flg) in LI samples show a more diffuse and slightly shifted staining pattern when compared with the normal samples. (xiii–xvi) Keratin 10 (K10) was expressed in suprabasal layers in all four samples. (**d** i–iv) The distributions of integrin-α6 (int-α6) and (v–viii) collagen VII (col VII) in LI skin/grafts in comparison with normal skin/grafts are visualized by immunostaining. These components of the dermoepidermal junction show a comparable staining in all samples, indicating a correct formation of the junction zone. Slides were counterstained with 4′,6-diamidino-2-phenyl indole. CC, cholesterol clefts.

give insights into molecular differentiation mechanisms (Table 1).

The numbers of unique peptides from identified proteins in the grafts were highly consistent with those found in human skin biopsies. No TG1 peptides could be detected in LI skin. The four TG1 peptides detected in one of three LI grafts (Table 1) were likely derived from surrounding mouse tissue, as the sequences are identical in mouse and human.

Notably, the number of unique filaggrin peptides decreased markedly in both LI skin and grafts. Profilaggrin is proteolytically processed during keratinocyte differentiation and subsequently degraded into hydrophilic amino acids, their metabolites, and ions that contribute to moisture retention in the SC. Expression of filaggrin and its hydrolysis into these natural moisturizing factors (NMFs) are influenced by the SC microenvironment, including local pH, external humidity, and transepidermal water loss (TEWL) (O'Regan *et al.*, 2008). The reduction of filaggrin detected by proteome analysis in LI skin/grafts may be attributed to an increased hydrolysis into NMFs to compensate for TEWL.

In contrast, the number of unique loricrin peptides increased in LI in comparison with normal skin/grafts. Loricrin becomes extensively cross-linked to numerous CE components by different TGs (Candi *et al.*, 2005). Insufficient intermolecular oligomerization by TG1 could result in an enhanced accessibility of trypsin cleavage sites during sample processing, resulting in an increase of unique peptides. Similarly, a marked increase of unique peptides of keratinocyte proline-rich protein (KPRP) was found in LI skin/grafts. KPRP expression was markedly increased in psoriatic lesions, suggesting that it could be extensively cross-linked by TG1 like small proline-rich proteins or some late CEs (Lee *et al.*, 2005).

Interestingly, we did not observe changes in the number of unique peptides of involucrin, one of the first proteins to be cross-linked to initiate CE assembly by forming a monomolecular

Table 1. Numbers of unique peptides of transglutaminase 1, cornified envelope components, and desmosome/corneodesmosome components identified by MS/MS-analysis in two or three independent samples derived from normal human skin, normal grafts, human LI skin, and LI grafts

		Number of unique peptides			
Protein	**MW (kDa)**	**Normal skin**	**Normal graft**	**LI skin**	**LI graft**
Transglutaminase 1	90	17, 10	10, 11	0, 0	0, 4[1], 0
Filaggrin	435	74, 110	76, 84	10, 13	13, 12, 16
Keratinocyte proline-rich protein	64	35, 39	35, 34	70, 59	51, 55, 53
Involucrin	68	16, 3	3, 11	15, 5	14, 5, 10
Loricrin	26	2, 2	0, 2	29, 24	16, 18, 22
Late cornified envelope protein 1C	12	2, 2	0, 0	5, 4	9, 8, 8
Small proline-rich protein 1B	10	0, 0	0, 0	2, 2	2, 2, 3
Desmoplakin	332	77, 28	40, 36	146, 128	135, 136, 137
Junctional plakoglobin	82	30, 14	13, 19	51, 38	55, 56, 53
Desmoglein 1	114	25, 19	14, 24	53, 43	45, 39, 44
Desmocollin 1	100	7, 0	0, 10	16, 17	12, 8, 10
Corneodesmosin	52	0, 0	0, 0	3, 4	2, 4, 3

[1]Four unique TG1-peptides (GSGVNAAGDGTIR; GTNPSAWVGSVEILLSYLR; YDTPFIFAEVNSDK; NPLPVTLTNVVFR) detected in only one sample are identical to mouse TG1-peptides.

layer adjacent to the cell membrane (Candi *et al.*, 2005). We speculate that this location does not alter its accessibility to trypsin.

Desmoplakin, a structural cytoskeleton constituent, which participates in keratinocyte adhesion, and other desmosomal/corneodesmosomal proteins such as desmoglein 1, desmocollin 1, junction plakoglobin, and corneodesmosin display a marked increase in unique peptide numbers in LI skin/grafts. From a clinical perspective, it is likely that increased expression of desmosomal adhesion molecules results in increased ''stickiness'' of corneocytes and thus explains why in LI the scales are large and plate-like, as normal, invisible desquamation did not occur. The rigid adhesion of cells is thought to be a compensatory effect to prevent TEWL (Steinert *et al.*, 1998).

We conclude that the LI skin-humanized mouse model faithfully recapitulates the human disease phenotype and concomitant molecular changes and can be used as an excellent tool for testing new therapeutic approaches for this up-to-now untreatable genodermatosis.

The study was approved by the Institutional Review Board of the University Hospital of Münster. All patients enrolled gave their written, informed consent. All animal studies have been approved by Centro de Investigaciones Energéticas Medioambientales y Tecnológicas's Institutional Review Board and all experimental procedures were conducted according to European and Spanish laws and regulations.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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