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**Towards *ex vivo* gene therapy for
Transglutaminase 1-dependent Lamellar Ichthyosis**

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“L’unica persona che sei destinato a diventare è la persona che tu decidi di essere.”

(John Lennon)

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Abstract

Lamellar ichthyosis (LI) is an autosomal recessive congenital ichthyosis (ARCI) characterized by generalized skin dryness, hyperkeratosis and appearance, since the first week of life, of coarse and brown/dark scales. LI is usually caused by mutations in *TGM1* gene, which encodes transglutaminase 1, an enzyme expressed in all keratinized stratified squamous epithelia and involved in skin barrier formation.

To date, no therapies targeting the molecular cause of the disease are still available and current therapies only focus on symptomatic relief. In this project, we propose to develop an *ex vivo* gene therapy approach for the treatment of LI patients, based on the generation of autologous cultured epidermal sheets containing genetically corrected epidermal stem cells. An integrating-viral vector with a physiological promoter has been developed to assess tissue- and stratum-specific expression of *TGM1* gene in primary keratinocytes. Organotypic skin cultures have been realized to evaluate the protein expression and the rescue of enzyme function during the keratinocyte differentiation process in a biomimetic, physiological environment. In addition, due to the rarity of the disease and the difficulties to retrieve skin biopsies, *TGM1* biallelic knockout was established using the CRISPR/Cas9 technology in human primary keratinocytes to generate a cellular model for LI. Such model has been employed for a proof of concept in preclinical research settings, waiting for primary clonogenic keratinocytes derived from LI patients. Gene-corrected *TGM1*-null keratinocytes displayed proper *TGM1* expression and enzymatic activity in the organotypic skin culture system, consistent with proper differentiative epidermal architecture. Such gene therapy approach demonstrated the restoration of cornified envelope formation comparable to level seen in normal keratinocytes, indicating functional correction of the proposed primary pathophysiologic defect in LI. This preliminary preclinical study supports a potential development of an *ex vivo* gene therapy approach to permanently regenerate a fully functional epidermis in patients suffering from this devastating skin disease.

1. INTRODUCTION

1.1 STRUCTURE OF EPIDERMIS

The mammalian epidermis is a stratified squamous epithelium that provides a protective barrier against harmful microorganisms and prevents the loss of essential body fluids, hence maintaining the internal milieu (Candi et al. 2005; Wickett and Visscher 2006; Fuchs 2016). The epidermis is tasked with this vital function while constantly self-renewing and healing wounds (Fuchs 2007; Moreci and Lechler 2020).

The human epidermis consists mainly of keratinocytes organized in distinct cellular layers (**Figure 1.1**) (Chu 2008). The basal layer represents the epidermal proliferative compartment and contains stem cells and transient-amplifying (TA) progenitors. As keratinocytes leave the basal layer and move toward the skin surface, they withdraw from the cell cycle and execute a terminal differentiation program, generating the suprabasal layers of epidermis (spinous layer, granular layer and cornified layer) (Watt 2002; Gambardella and Barrandon 2003; Blanpain and Fuchs 2006; Fuchs 2007; Blanpain and Fuchs 2014). This process, known as cornification or keratinization, envisages a genetically programmed series of morphologic and metabolic changes whose endpoint is the generation of the outermost layer of epidermis: the cornified layer or stratum corneum (SC) (Candi et al. 2005; Chu 2008).

Stem cells of basal layer

Clonogenic keratinocytes, residing in the epidermal basal layer, form a single cell layer firmly attached to the basement membrane zone that separates the epidermis from the underlying dermis (**Figure 1.1**) (Christiano and Uitto 1996; Lian and Murphy 2015).

Three types of human epidermal clonogenic keratinocytes have been identified, which give rise to clones referred to as holoclones, meroclones and paraclones (Barrandon and Green 1987). Although all clonal types retain proliferative capacity, recently it has been definitely demonstrated that the holoclone-forming cell is the only clonal type possessing self-renewing properties and long-term regenerative potential, being the stem cell of all squamous epithelia (Rama et al. 2010; Hirsch et al. 2017). Generation of all specialized keratinocytes occurs through a phenomenon called clonal conversion, in which holoclones, generate meroclones and paraclones that ultimately produce terminally differentiated keratinocytes. Holoclone-forming cells are long-lived, self-renewing epidermal stem cells, while meroclone- and paraclone-forming cells are short-lived progenitors progressively lost

during epidermal renewal, but essential for proper regeneration, wound healing, and stratification of the epidermis (Hirsch et al. 2017).

A few molecular markers highly expressed in human holoclones and progressively decreasing during clonal conversion have been identified. The transcription factor p63 (Mills et al. 1999; Yang et al. 1999), and in particular the $\Delta Np63\alpha$ isoform, underpins the proliferative, regenerative capacity of squamous epithelial stem cells. Its expression decreases during clonal conversion from holoclone to meroclone and is lacking in paraclones (Pellegrini et al. 2001; Senoo et al. 2007). Indeed, measurements of $\Delta Np63\alpha$ abundance in holoclones can be used to estimate the number of stem cells in a limbal culture (Pellegrini et al. 2001; Rama et al. 2010). Another important factor enriched in human epidermal holoclone-forming cells is the Yes-associated protein (YAP). YAP is a transcriptional co-activator that regulates the proliferation of a multitude of stem and progenitor cells. When YAP is de-phosphorylated, it translocates from the cytoplasm to the nucleus where it interacts with the transcription factor TEAD, activating its target genes. In holoclones, unphosphorylated YAP interacts with $\Delta Np63\alpha$ allowing the maintenance of self-renewal and proliferative/regenerative capacity of human epidermal stem cells. (De Rosa et al. 2019). Another molecular determinant that has been associated to holoclones is FOXM1, which belongs to the forkhead box family and acts downstream of YAP (Enzo et al. 2021). As observed with YAP, ablation of FOXM1 in a human epidermal culture leads to holoclone depletion, while enforced FOXM1 halts clonal conversion and extends the keratinocyte life-span indefinitely. The combination of all these markers contributes to define the molecular signature of human epidermal holoclones.

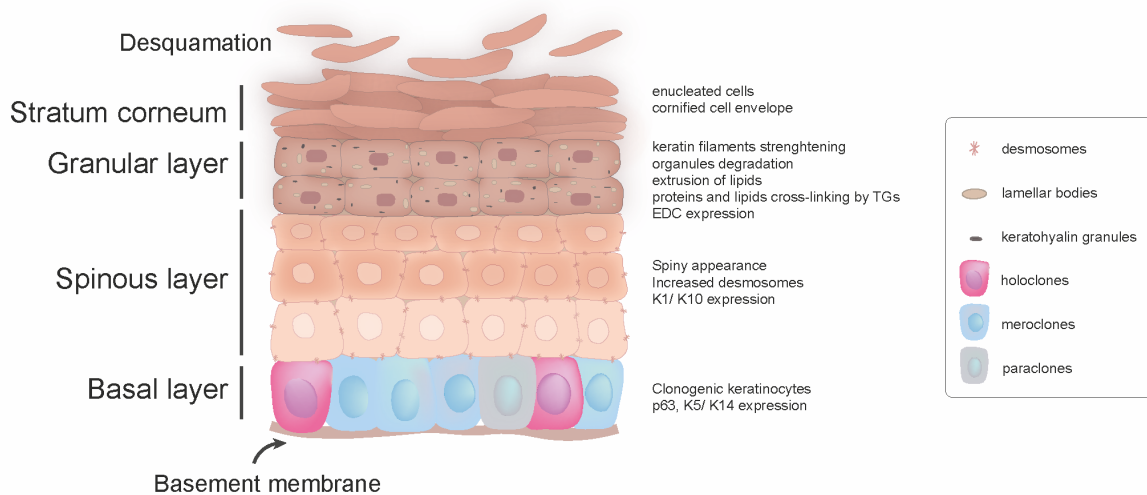


Figure 1.1. Basic structure of the human epidermis. Human epidermis consists of keratinocytes organized in different layers (indicated on the left). Holoclones (magenta), meroclones (light blue) and paraclones (grey) are clonogenic keratinocytes located in the basal layer, firmly attached to the basement membrane. Physical characteristics and key molecular markers of each layer are indicated on the right.

Epidermal keratinization, barrier formation

The process of epidermal keratinization and cornification is a unique form of programmed cell death, regulated by several signaling molecules. Such process begins in the basal layer of the epidermis, generates several layers of terminally differentiated keratinocytes and culminates in the formation of the cornified envelope (CE) of the stratum corneum (SC), which consists of keratins embedded in an insoluble blending of proteins and lipids (Candi et al. 2005; Chu 2008; Kolarsick et al. 2011). As in a “dynamic flux”, during epidermal homeostasis, cell proliferation in the basal layer is balanced by desquamation of the cornified layer at the skin surface, hence the epidermis self-renews approximately every 2 months throughout life (Fuchs and Raghavan 2002; Chu 2008; McLafferty et al. 2012; Lian and Murphy 2015).

As keratinocytes migrate from the basal layer, they undergo characteristic morphological and biochemical changes. Through the spinous layer they first become polyhedral and progressively flatter, stop their mitotic activity and synthesize differentiation-specific enzymes and structural proteins. Keratin 1 (K1) and keratin 10 (K10) are among the first proteins to be expressed during terminal differentiation, replacing the pre-existing keratin 5 and keratin 14 forming the intermediate filaments (IF) network of basal keratinocytes (Ling-juan 2018). Spinous keratinocytes express involucrin, the first structural component of the forthcoming epithelial barrier. In fact, most of proteins forming the barrier are synthesized

during the keratinization process and are encoded by genes located in the epidermal differentiation complex (EDC) locus, on human chromosome 1q21 (Candi et al. 2005; Miller et al. 2008). Upper spinous cells are also responsible for the synthesis and accumulation of lipids in well-organized structures termed *lamellar bodies*, which accumulate to higher levels in the granular cells. Lamellar bodies are secretory organelles containing precursors of the stratum corneum lipids such as glucosyl ceramides, cholesterol, cholesterol esters, and long-chain fatty acids, all of which are secreted into the intercellular space of the granular and cornified layers forming the main hydrophobic barrier to water passing out through the SC (Kolarsick et al. 2011).

Being the last layer of epidermis containing living cells, the granular layer represents the site where the key events leading to the formation of the epithelial barrier occur. This layer is made of flattened cells characterized by the presence of cytoplasmic keratohyalin granules. Composed primarily of involucrin, profilaggrin, loricrin and keratin filaments, these granules result from the accumulation of newly synthesized proteins that will be the structural and flexible components of the epidermal barrier (Steinert and Marekov 1995; Candi et al. 2005; Eckhart et al. 2013). In particular, profilaggrin undergoes calcium-dependent cleavage and dephosphorylation to form filaggrin, which acts as a matrix glue fostering macromolecular organization of intermediate filaments into larger bundles (Chu 2008). Filaggrin rapidly aggregates the keratin cytoskeleton, eventually causing collapse of the granular cells into flattened anuclear squames in the SC (Deo and Deshmukh 2018). In fact, granular keratinocytes execute the process of programmed cell death that leads to the generation of terminally differentiated anucleated corneocytes, which are devoid of all cellular components. Thus, the cornified layer is an organized structure of anuclear, flattened cells, stacked like “bricks in a wall” (Elias 1983; Harding 2004; Wickett and Visscher 2006), which provides physical protection to the skin and a barrier to water loss and permeation of foreign substances from the environment. This crucial function of the cornified layer is fulfilled by the cell envelope (CE), a 10 nm thick stratum of highly crosslinked insoluble proteins that is assembled underneath, and eventually replaces, the corneocyte plasma membrane (**Figure 1.2**) (Kalinin et al. 2001; Candi et al. 2005; Chu 2008; Kolarsick et al. 2011; Lian and Murphy 2015; Moreci and Lechler 2020). An additional 5nm thick ‘ceramide lipid envelope’, covalently attached to the outer CE surface, significantly contributes to the establishment of epithelial elasticity and barrier function (**Figure 1.2**) (Elias 1983; Harding 2004; Elias et al. 2014).

The high rigidity of the CE is due to specific transglutaminases (TGs), which are Ca^{2+} -dependent enzymes that catalyze the formation of N ϵ -(γ -glutamyl) lysine bonds between different proteins. Six TGs are expressed in the epidermis, but only TG1, TG3 and TG5

contribute to CE formation (Lorand and Graham 2003). These enzymes are synthesized in the upper spinous layer but remain inactive until they encounter the high levels of calcium in the upper granular/cornified layers. Particularly, during the first stage of the CE formation, TG1 and TG5 crosslink envoplakin, periplakin and involucrin under the cell membrane, forming a monomolecular layer that acts as a scaffold for further assemblies (**Figure 1.2**) (Kalinin et al. 2002; Harding 2004; Candi et al. 2005). In the second step, perhaps coincident with initiation of scaffold assembly, various lipids (fatty acids, cholesterol and its esters, and ceramides) are secreted from lamellar bodies of the granular layer and packaged on the outer surface of the plasma membrane, forming lamellae between the corneocytes of the cornified layer. Moreover, specialized ω -hydroxy-ceramides are ester-linked by TG1 on the already crosslinked proteins (envoplakin, periplakin, involucrin), forming the outer lipid envelope (Nemes et al. 1999; Kalinin et al. 2002; Eckhart et al. 2013). As the last step in CE formation, TG1 crosslinks loricrin and small proline-rich proteins (SPRs) to the already built CE scaffold. Loricrin acts as a main reinforcement protein for the cornified envelope on its cytoplasmic face, accounting for 70-85% of the total protein mass of the cornified layer (Kalinin et al. 2002). Minor components of the complex CE structure include repetin, trichohyalin, cystatin A, elafin and LEP/XP-5 proteins become crosslinked to the CE (Steven and Steinert 1994; Steinert and Marekov 1995; Kalinin et al. 2002). The resulting resistant but flexible layer of dead cells embedded in the lipid lamellae provide the vital mechanical and water permeability barrier functions.

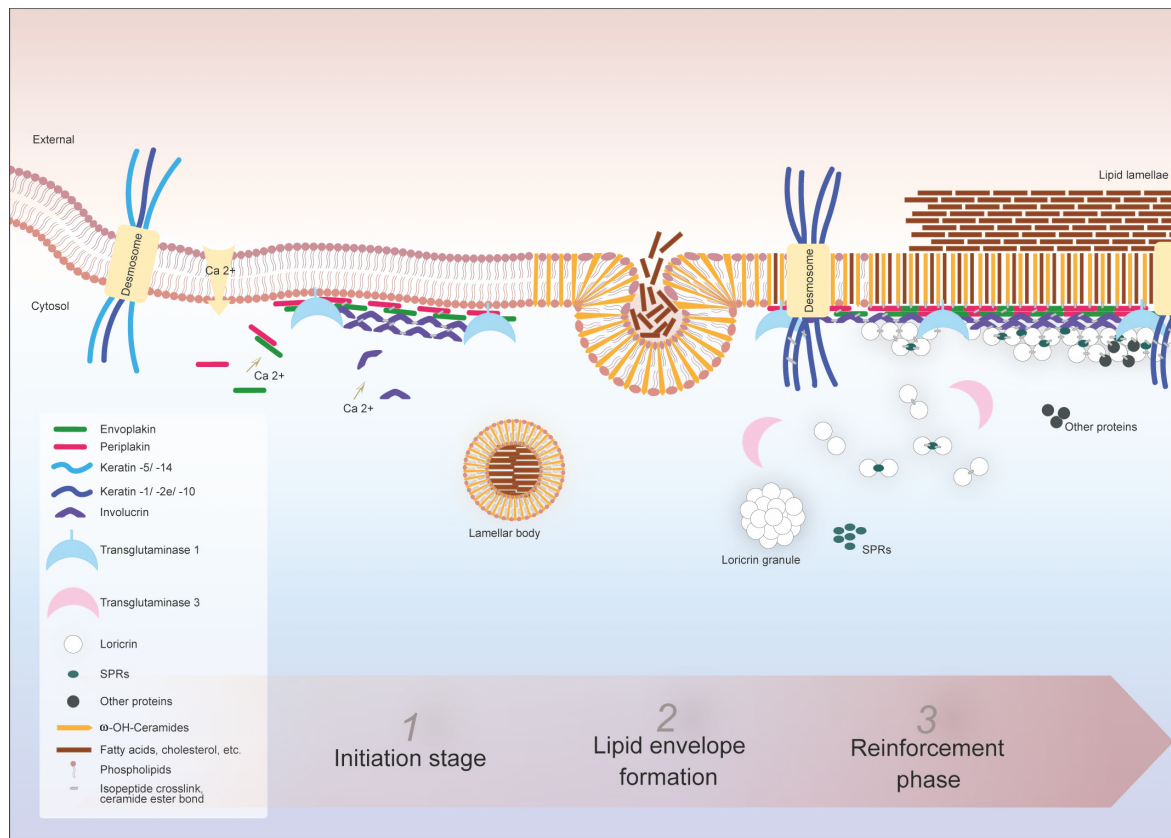


Figure 1.2. Cornified Envelope assembly. CE assembly is a precisely regulated and orderly process triggered by a rise of intracellular Ca^{2+} concentration coincident with signals to initiate terminal differentiation in stratified squamous epithelia. The assembly could proceed in three stages. During the initiation stage, which takes place in the spinous layer, envoplakin (green) and periplakin (red) associate with keratin intermediate filaments (blue and light blue) and desmosomal junctions. TG1 (Transglutaminase 1) and involucrin (violet) expression begins shortly after and they spontaneously associate with the membrane. A monomolecular layer of involucrin, envoplakin and periplakin forms along the entire inner surface of the cell membrane, including over desmosomes, forming a 'scaffold' for further assembly. Next, during the formation of the lipid envelope, in the granular layer, lipids from the lamellar bodies derived from the Golgi (ω -OH-ceramides, brown and fatty acids, ochre), are attached and crosslinked by TG1 on the already crosslinked proteins and are exposed on the outside of the membrane. The released lipids fill the inter-corneocyte spaces, resulting in a lipid barrier preventing water loss from epidermis. Last, the reinforcement phase occurs, which involves the further crosslinking of loricrin, SPRs and other proteins by TG1 on the protein scaffold, at the intracellular side. Together, the complete CE (protein and lipid envelope components) and intercellular lipid lamellae provide the essential physical and water barrier functions for the skin and entire organism.

1.2 INHERITED DISORDERS OF CORNIFICATION

Mendelian disorders of cornification (MeDOC) refer to a large, heterogeneous group of diseases involving the entire integument (Elias et al. 2010a; Oji et al. 2010) and caused by defects in the formation of the SC and/or CE. MeDOC are clinically characterized by generalized skin dryness, hyperkeratosis and scaling, often associated with erythroderma (Elias et al. 2010a; Arkin et al. 2015).

Inherited ichthyoses are a group of MeDOC caused by mutations in over 30 genes encoding a wide spectrum of epidermal proteins, including i) enzymes governing lipid metabolism or peptide cross-linking, ii) proteases and their inhibitors, iii) epidermal structural proteins, and iv) proteins involved in cellular communication, signaling and gene transcription. Abnormalities in any of these proteins result in impairments of the skin barrier function and consequent epidermal hyperplasia, hyperkeratosis and visible accumulation of squames (scales) on the skin's surface – the clinical hallmark of all the ichthyoses – (Schmuth et al. 2007; Elias et al. 2010b; Vahlquist et al. 2018; Hernández et al. 2019). Such condition causes itching, inflammation and pain, drastically reducing the quality of life of patients. To date, there is still no cure for this disease and current therapies only help to improve symptoms (Fleckman et al. 2013; Cortés et al. 2020).

Ichthyoses classification

According to the first international ichthyosis consensus conference of 2009 in Sorèze, two main disease groups have been recognized based on phenotype manifestations (**Table 1.1**) (Oji et al. 2010). Non-syndromic ichthyoses are characterized by a phenotypic expression limited to the skin, while syndromic ichthyoses present significant extracutaneous manifestations (e.g., Netherton syndrome). Non-syndromic ichthyoses include Ichthyosis Vulgaris (IV), Recessive X-linked Ichthyosis (RXLI), Autosomal Recessive Congenital Ichthyosis (ARCI), Keratinopathic Ichthyosis (KI) and other forms (Oji et al. 2010).

		Disease	Inheritance	Gene(s)	Function
Inherited Ichthyoses	Nonsyndromic forms	Common ichthyoses			
		Ichthyosis vulgaris (IV)	ASD	FLG	Keratin aggregation, SC hydration
		Recessive X-linked ichthyosis (RXLI)	XLR	STS	SC lipid composition
		Autosomal recessive congenital ichthyosis (ARCI)			
		<i>Major types</i>			
		Harlequin ichthyosis (HI)	AR	ABCA12	Lipid transport and secretion
		Lamellar ichthyosis (LI)	AR	TGM1 in most, ALOX12B, ALOXE3, ABCA12, CYP4F22, NIPAL4/ICHTHYIN, CERS3, PNPLA1	CE cross-linking, lipid composition and attachment, signaling
		Congenital ichthyosiform erythroderma (CIE)	AR	TGM1, ALOX12B, ALOXE3, ABCA12, CYP4F22, LIPN, NIPAL4/ICHTHYIN, CERS3, PNPLA1	"
		<i>Minor types</i>			
		Self-healing collodion baby (SHCB)	AR	TGM1, ALOX12B, ALOXE3	"
		Bathing suit ichthyosis (BSI)	AR	TGM1	CE cross-linking
		Keratinopathic ichthyosis (KPI)*			
		Epidermolytic ichthyosis (EI)	AD	KRT1, KRT10	Mechanical stability of keratinocytes
		Superficial epidermolytic ichthyosis (SEI)	AD	KRT2	"
		Other forms			
		Loricrin keratoderma (LK)	AD	LOR	Corneocyte stability, scaffold function
		Erythrokeratoderma variabilis (EKV)	AD	GJB3, GJB4	Calcium regulation
		Peeling skin disease (PSD)	AR	CDSN	corneodesmosomes structure
		Keratosis linearis with ichthyosis congenita and keratoderma (KLICK)	AR	POMP	Proteasome insufficiency
	Syndromic forms*	X-linked ichthyosis syndromes (delayed onset)	XLR	STS	SC lipid composition
		Netherton syndrome (NS)	AR	SPINK5	Stratum corneum desquamation
		Refsum syndrome	AR	PHYH, PEX7	Fatty acid metabolism
		Sjögren-Larsson syndrome (SLS)	AR	ALDH3A2	"
		Neutral lipid storage disease with ichthyosis	AR	ABHD5	"
		Trichothiodystrophy syndromes (TTD)	AR	C7ORF11, ERCC2/XPD, ERCC3/XPB	DNA transcription, excision repair

Table 1.1. Clinicogenetic classification of Inherited Ichthyoses. Abbreviations: AD, autosomal dominant; AR, autosomal recessive; ASD, autosomal semi-dominant. *Only major types are described. *Only some examples are mentioned.

Lamellar Ichthyosis (TGM1 Mutations)

To date, ARCI have been associated with mutations in 8 genes which encode proteins involved in lipid biosynthesis and transport, fatty acid metabolism, or CE formation (Takeichi and Akiyama 2016; Vahlquist et al. 2018; Hernández et al. 2019). Lamellar Ichthyosis (LI) is one of the most severe form of ARCI. Over 30% of LI is caused by mutations in *TGM1*, the gene encoding TG1, which is located on chromosome 14q11.2 (Russell et al. 1994; Huber et al. 1995; Farasat et al. 2009; Fischer 2009; Vahlquist et al. 2018).

Clinically, LI is characterized by severe skin dryness, erythema, hyperkeratosis and massive scaling, all of which highly impair patients' quality of life. Large, plate-like dark-brown scales cover the entire body surface, and variable underlying erythema is present (Fleckman and DiGiovanna 2012; Schmuth et al. 2013; Takeichi and Akiyama 2016). Affected individuals are often born with a 'collodion membrane' that sheds in the first week of life leaving patients with a disfiguring ichthyosis, detectable with coarse and brown/dark scales over the whole body (Schmuth et al. 2007; Takeichi and Akiyama 2016). Loss of TG1 function results in an impaired epidermal barrier with dramatically increased transepidermal water loss (TEWL), causing severe, often life threatening, dehydration, especially during the first weeks of life. Depending on *TGM1* mutations, LI severity ranges from mild to severe (Raghunath et al. 2003; Akiyama and Shimizu 2008; Mazereeuw-Hautier et al. 2009; Hackett et al. 2010; Oji et al. 2010; Bourrat et al. 2012; Arkin et al. 2015; Hernández et al. 2019), with ectropion and eclabium (reversal of the eyelids and lips, as a result of tension from the collodion membrane) and scarring alopecia. Flexural surfaces and palms and soles are frequently involved (Fleckman and DiGiovanna 2012; Arkin et al. 2015).

TGM1 gene and its pathogenic variants

The *TGM1* gene consists of 14,064 bp, with a 2,454 bp coding sequence spanning over 15 exons (ENST00000206765.11). The protein product of *TGM1* is Transglutaminase-1 (TG1), a 90 kDa membrane-bound enzyme which is made of up to 817 amino acid residues. It is a Ca^{2+} -dependent crosslinking enzyme that catalyzes the formation of N ϵ -(γ -glutamyl)lysine bonds between (and within) proteins during the formation of the CE (Abernethy et al. 1977; Rice and Green 1977). In particular, the γ -carboxamide group ($-(\text{C}=\text{O})\text{NH}_2$) of a glutamine residue and the ϵ -amino group ($-\text{NH}_2$) of a lysine residue are covalently bound by TG1, giving rise to stable protein polymers that provide the characteristic insolubility of the CE in detergent and reducing agents (Rice and Green 1977). Furthermore, TG1 can form ester-linkages between epidermal specific ω -

hydroxyceramides and several CE structural proteins (most notably Involucrin), to form the corneocyte lipid envelope (Nemes et al. 1999).

The full-length TG1 apoprotein is usually inactive. During maturation, TG1 is proteolyzed in three different fragments of 10-, 67- and 33- kDa (Steinert et al. 1996) that remain associated in the active membrane-bound form (Candi et al. 2005). TG1 enzymatic activity, which is known to require calcium as a cofactor, becomes upregulated as a result of epidermal differentiation. In the absence of a TG1 X-ray crystal structure, molecular modeling was used to generate a predicted structure of TG1. The protein is expected to consist of five domains: (i) the N-terminal residue that contains the 10 kDa membrane anchorage region, (ii) the β -sandwich domain and (iii) catalytic core domain both comprising the 67 kDa fragment, (iv) the β -barrel 1 and (v) β -barrel 2 domains which comprise the 33 kDa fragment (**Figure 1.3**) (Lorand and Graham 2003; Boeshans et al. 2007).

More than 100 different pathogenic variants in TG1 have been identified. The majority of them are single-base mutations, but also insertions or deletions have been found. *TGM1* pathogenic variants include primarily missense, non-sense and splice site mutations. Most pathogenic variants are located in the first two-thirds of the gene, comprising the catalytic core domain of the enzyme, and often lead to truncated proteins (**Figure 1.3**) (Farasat et al. 2009; Herman et al. 2009).

Indeed, decreased to absent enzyme activity has been reported in several patients with *TGM1* germline mutations (Raghunath et al. 1998; Cserhalmi-Friedman et al. 2001; Akiyama et al. 2003; Mazereeuw-Hautier et al. 2009). Strikingly, several reported pathogenic variants have been found in highly conserved amino acids, for example in arginine residues. Arg residues are highly mutable due to the deamination of 5'-CpG dinucleotides resulting in C $\rightarrow$ T or G $\rightarrow$ A transitions, which has been found in about 40% of all *TGM1* mutations (Farasat et al. 2009). Other *TGM1* variants can reduce mRNA stability and/or promote the activation of nonsense mediated mRNA decay (NMD) (McIntosh et al. 1993; Maquat 2004), ultimately leading to complete TG1 ablation (Herman et al. 2009). The most common pathogenic variant of *TGM1* affects the intron 5 splice acceptor site (c.877-2A>G; alias IVS5-2A>G) and has been found in approximately 34% of LI patients worldwide (Huber et al. 1995; Hennies et al. 1998; Pigg et al. 1998; Shevchenko et al. 2000; Farasat et al. 2009; Esposito et al. 2013). Analysis of mRNA from patients with c.877-2A>G showed that the splice-site mutation leads to two different splice variants, both of which result in a premature stop codon (Huber et al. 1997; Shevchenko et al. 2000).

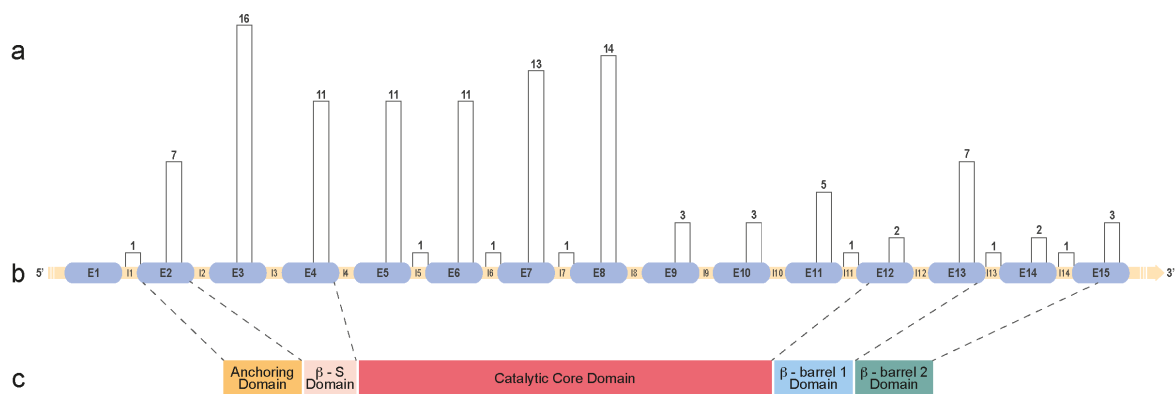


Figure 1.3. *TGM1* gene and its pathogenic variants. a) Distribution of *TGM1* mutations reported in the literature. b) *TGM1* gene structure. c) TG1 protein domains.

1.3 DIAGNOSIS AND CLINICAL MANIFESTATION

To date, a molecular diagnosis is increasingly feasible in patients with inherited ichthyosis and is a prerequisite for giving correct genetic advice and information about prognosis and treatment. Despite recent efforts in understanding the pathophysiology of this debilitating disease, so far there is no curative therapy available and only symptomatic relief can be provided (DiGiovanna et al. 2013; Fleckman et al. 2013; Cortés et al. 2020). Inherited ichthyoses represent life-long conditions which greatly influence the appearance and physical state of the affected individuals. However, there is a large spectrum of disease expression, ranging from very mild to more severe with variable degrees of scaling, hyperkeratosis, and erythema, which require different approaches and even individualized therapies.

In infants suffering of severe collodion membrane at birth, temperature instability and dehydration are functional consequences of the epidermal barrier defect. Indeed, neonates often require intensive care using incubators and balneotherapy, and vigilance for signs of cutaneous infection and septicaemia (Oji and Traupe 2009; Oji et al. 2010; Hernández et al. 2019). Growth failure is present in several forms of ichthyosis due to the impaired permeability barrier and heat evaporation, both causing a significant increase in energy consumption (Moskowitz et al. 2004). Ocular complications, including scales on the eyelashes and ectropion, are common in all types of severe ichthyosis and may eventually lead to corneal damage. Indeed, in more severe cases of ectropion, repeated skin transplantations are often needed (Vahlquist et al. 2018). Abnormalities of the nails, hair

and mucous membranes are characteristic of several subtypes, for example trichorrhhexis in Netherton Syndrome or alopecia in Keratitis Ichthyosis (Schmuth et al. 2013). Although some types of inherited ichthyoses may show spontaneous and seasonal variations, most patients will require a daily therapy throughout life, with soaking bath followed by mechanical scales removal (Oji and Traupe 2009). Severely affected patients often need 1–2 hours or more for their daily skin treatment. Other common symptoms are pruritus, fissuring, sweating impairment (hypohidrosis) and superinfections.

Moreover, for syndromic ichthyoses cutaneous manifestation is not the only clinical feature of a multi-system disorder which also presents central nervous system and digestive system involvement (for example in Netherton Syndrome) (Schmuth et al. 2013). Of note, the range and severity of symptoms usually vary considerably from one patient to another and may also change over time in the same patient. This makes it essential to adjust therapy according to the individual and current needs of the patient unless long-term therapy is available.

1.4 STATE OF THE ART ON THERAPEUTICAL APPROACH FOR ICHTHYOSIS

Currently, treatments of most inherited ichthyosis are rarely type-specific and corrective. Rather, therapies are symptomatic and mainly focused on: (i) maintain or improve barrier function (prevent transepidermal water loss and facilitate desquamation), (ii) ameliorate cutaneous appearance and comfort, as well as (iii) treat secondary complications (e.g., skin infections). Since mainly ichthyoses are chronic life-long diseases, there is a need for well-tolerated, safe, and effective therapies. Nowadays, treatments of choice are ointments or drugs administered through topical or systemic approaches, but also efforts have been made to develop more tailored treatments, such as the enzyme replacement therapy and the cutaneous *ex vivo* and *in vivo* gene therapy.

Topical treatments

Topical therapy is aimed at restoring the epidermal barrier, facilitating desquamation, and improving the overall cutaneous appearance of the treated patients. Diverse agents or their mixes can be employed to carry out these tasks (Oji and Traupe 2009; Vahlquist et al. 2018; Cortés et al. 2020). Some of these molecules, including urea (<10%), glycerol or dexpanthenol, act as humectant agents by increasing the water content in the stratum corneum to improve skin hydration. Other compounds can instead promote exfoliation of corneocytes by reducing cellular cohesion through a keratolytic effect, as with lactic acid, urea (>10%), glycolic acid, salicylic acid, or retinoids. Further molecules, such as lanolin or

petrolatum (Cortés et al. 2020), can directly reduce TEWL, being bland emollients that create an occlusive lipid layer on the skin surface that prevent moisture loss. However, the risk of systemic absorption of the ingredients, especially for patients treated over the whole body, must always be considered. Indeed, high blood concentrations of these drug are dangerous, and can lead to severe adverse events (Schmuth et al. 2013; Vahlquist et al. 2018).

Systemic treatments

Systemic therapy is usually reserved for severe forms of ichthyosis that do not respond adequately to topical therapy. Being this therapy a long-term, and often life-long treatment, expected outcomes and potential side-effects must be widely considered. Systemic therapy for ichthyosis is usually based on oral retinoids, analogs of all-trans retinoic acid, vitamin A derivative, such as etretinate (which is no longer commercially available in most European countries), acitretin and isotretinoin (Vahlquist et al. 2018; Hernández et al. 2019; Cortés et al. 2020). The mechanism of action of retinoids is complex and still not quite clear. Has been demonstrated that retinoids act as ligands for nuclear transcription factors that regulate numerous genes and hence modulate keratinocyte protein expression. Indeed, several retinoids can modify epidermal proliferation, differentiation, and facilitate desquamation through down-regulation of protein components of corneodesmosomes (Törmä 2011; DiGiovanna et al. 2013). Consequently, retinoids improve scaling, hyperkeratosis, hair regrowth, and hypohidrosis. However, they tend to increase skin fragility and are only effective for as long as they are used. It is important to note that these drugs also cause severe adverse effects, including teratogenesis and reduction of skeletal growth, which must be considered for the treatment of women of childbearing age and children. Furthermore, retinoids are lipophilic drugs that are slowly eliminated from the body (DiGiovanna et al. 2013; Vahlquist et al. 2018; Cortés et al. 2020).

An alternative for systemic treatment is the new class of drugs called “retinoic acid metabolism-blocking agents” (RAMBAs). One of them, namely liarozole, has been granted orphan drug status for the treatment of congenital ichthyosis by the EMA and the FDA. Liarozole inhibits the catabolism of endogenous retinoic acid thus increasing its levels in skin (Oji and Traupe 2009). However, the advantage of oral liarozole over acitretin is uncertain (Hernández-Martin et al. 2013; Vahlquist et al. 2014).

Enzyme replacement therapies

Topical protein delivery allows for convenient and pain-free self-administration for patients and thus increases compliance, however, delivery through the skin has always been an

attractive as well as challenging area in most cases impracticable. Indeed, foreign substances, for example macromolecules, face several extracellular and intracellular barriers that must be overcome before generating any therapeutic effect. Consequently, non-viral delivery systems have been designed to prevent the degradation of genetic material through endonucleases, escape immune detection, and reduce nonspecific interactions (Zeng et al. 2021). Recent experimental studies in the field of enzyme replacement therapy (ERT) for ichthyosis are based on nanoparticles and liposomes delivery.

Cationic liposomes with encapsulated recombinant human TG1 (rhTG1) were produced to develop a topical approach suitable for restoring TG1 activity in the upper stratified layers of the epidermis. Using an ARCI skin-humanized mouse model, it was shown that after 2 weeks of topical applications with the rhTG1 liposomes, the ichthyosis phenotype was normalized, with no significant adverse effects (Aufenvenne et al. 2013). In a similar approach, TG1 enzyme delivery into 3D skin equivalents was mediated by use of thermoresponsive nanogels (tNG). ARCI 3D skin equivalents models were generated from fibroblasts plus keratinocytes from ARCI patients with *TGM1* mutations, and subsequently treated with TG1 loaded in tNGs, four times over 8 days. The authors reported that the enzyme-loaded nanogel efficiently delivered TG1 into ARCI 3D skin models, and seemingly improved barrier function through reduction of permeability of foreign substances (Plank et al. 2019).

Notably, although these approaches are attractive and technically feasible, they need to be repeated over time because the therapeutic effect still remains temporary.

Cutaneous ex vivo and in vivo gene therapy approaches

The first ex vivo gene therapy approach reported for LI was in 1996 (Choate et al. 1996b), with the delivery of the *TGM1* gene into LI patient cells, using an amphotropic retroviral vector. In brief, starting from *in vitro* transduced LI patient cells, Paul Khavari and his group were able to regenerate human epidermis on immunodeficient mice, demonstrating the possibility to correct the molecular, histologic, and functional abnormalities of LI patient skin.

Shortly thereafter, the same approach was used on keratinocytes from X-linked ichthyosis (Freiberg et al. 1997). Although long-term efficacy was not established at the time, both of these studies demonstrated the potential future approach for a therapeutic ex vivo gene therapy for skin diseases. Despite nowadays above one thousand clinical trials involve gene therapy, only two are focused on congenital ichthyosis.

The first (NCT01545323) started in 2018, is a phase I study of *ex vivo* lentiviral gene therapy for the Netherton syndrome. The *ex vivo* approach is based on the generation of autologous skin sheet graft obtained from *SPINK5*-corrected patient's cells, that will be subsequently regraft to the patient. This combined *ex vivo* cell and gene therapy would meet the remarkable goal of achieving a definitive, permanent restoration of the skin, but it has many drawbacks: the preparation of transgenic grafts is expensive and needs highly qualified centers. Furthermore, the transplantation of the cultures requires invasive surgical procedures, which, however, would not be repetitive for the treated areas.

The second (NCT04047732), started in 2020, is a phase I/II clinical trial of a topical gel (namely KB105) containing a replication-incompetent, non-integrating herpes simplex virus type 1 (HSV-1) vector expressing human *TGM1*, for the treatment of *TGM1*-deficient ARCI patients (Freedman et al. 2021). This strategy seems quite appealing, since it would not require expensive, cumbersome, and time-consuming manufacturing processes or invasive clinical procedures. Noteworthy, the episomal non-integrating nature of HSV-1 makes repeated—perhaps life-long—application necessary.

2. MATERIALS AND METHODS

2.1 CELL CULTURE AND MAINTENANCE

3T3-J2 cell line. Mouse 3T3-J2 cells were a gift from H. Green, Harvard Medical School. 3T3-J2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% irradiated calf serum, glutamine (4 mM) and penicillin–streptomycin (50 IU/ml).

Human primary keratinocytes. Human primary keratinocytes were retrieved from biopsies of healthy adult donors (K82 and K81 strains). A skin biopsy was minced and trypsinized (0.05% trypsin and 0.01% EDTA) at 36.5°C for 3h. Keratinocytes were collected every 30 min and plated (3×10^4 cells per cm^2) onto lethally irradiated 3T3-J2 cells (3×10^4 cells per cm^2) at 36.5°C with 5% CO_2 and humidified atmosphere in growth KNO medium (DMEM and Ham's F12 nutrient mixture (60% and 30%) containing irradiated fetal bovine serum (FBS) (10%), penicillin-streptomycin (50 IU/ml), glutamine (4 mM), adenine (0.18 mM), insulin (5 $\mu\text{g/ml}$), hydrocortisone (0.4 $\mu\text{g/ml}$), cholera toxin (0.1 nM), triiodothyronine (2 nM)). After 3 days KNO medium was replaced with KC medium (KNO medium containing EGF (10 ng/ml)). Keratinocytes were trypsinized (0.05% trypsin and 0.01% EDTA) at sub-confluence at 36.5°C for 15-20 min and replated (6×10^3 cells per cm^2) onto a new feeder-layer (3×10^4 cells per cm^2) in KNO medium, swapped with KC after 3 days. Sub-confluent cultures were frozen in liquid nitrogen (1×10^6 cells per vial).

Keratinocytes freezing procedure. Sub-confluent keratinocytes cell cultures were trypsinized and resuspended in KNO medium supplemented with 10% glycerol. Collected keratinocytes were aliquoted and frozen at -80°C. 24h later, cells were relocated in liquid nitrogen for long-term storage.

Feeder layer depletion. To remove feeder-layer cells from human primary keratinocytes cell cultures, warm PBS 1X supplemented with 20mM EDTA was added and pipetted over the cells until all feeder-layer was removed. Exhaust solution was eliminated, and cells were washed once with PBS.

HaCaT cells. HaCaT cells, spontaneously immortalized human keratinocyte line, were cultured in 5% CO_2 at 37°C in three different culture media as needed. *Standard culture medium:* DMEM supplemented with 10% heat-inactivated fetal bovine serum, glutamine (2mM), and penicillin-streptomycin (100 IU/ml). *Low Calcium medium (LC):* Keratinocyte Serum Free Media (KSFM) (Thermo Fisher) supplemented with 0.03 mM of Calcium Chloride, 25 $\mu\text{g/ml}$ Bovine Pituitary Extract (BPE, Thermo Fisher), 0.2 ng/ml of recombinant Epidermal Growth Factor (Thermo Fisher) and penicillin-streptomycin (100 IU/ml). *High Calcium medium (HC):* Keratinocyte Serum Free Media (KSFM) (Thermo Fisher)

supplemented with 2.8 mM of Calcium Chloride, 25 µg/ml Bovine Pituitary Extract (BPE, Thermo Fisher), 0.2 ng/ml of recombinant Epidermal Growth Factor (Thermo Fisher) and penicillin-streptomycin (100 IU/ml).

HaCaT freezing procedure. 80%-confluent HaCaT cell cultures were trypsinized (Trypsin 2.5 % in HBSS w/o Phenol Red w/o Calcium w/o Magnesium, Euroclone) at 37°C for 15 min and resuspended in one of the culture media supplemented with 10% glycerol, depending on the experiment. Collected HaCaT cells were aliquoted and frozen at -80°C. 24h later, cells were relocated in liquid nitrogen for long-term storage.

2.2 PLASMID CONSTRUCTS

Genomic DNA extraction. QIAmp DNA Mini Kit (Qiagen) was used to isolate genomic DNA from human primary keratinocytes (K81) in accordance with the manufacturer's instructions.

Construction of three different promoters to drive the expression of eGFP reporter gene. Genomic DNA from human primary keratinocytes (K81) was used as the template to generate the 637-bp human Involucrin minimal promoter (hIVL-min), the 367-bp human *TGM1* minimal promoter (h*TGM1*-min) and the 2.2-Kb human *TGM1* full length promoter (h*TGM1*-full), by polymerase chain reaction (PCR) using high fidelity Taq polymerase (LA Taq DNA polymerase, Takara). Specific primers were used to amplify the distal region (-2.473 Kb / -2.036 Kb) and the proximal region (-221 bp / +6 bp) of wild type human involucrin promoter (Ghazizadeh et al. 2002), the distal region (-1.6 Kb / -1.4 Kb) (containing the AP1 and Sp1 binding sites) and the proximal region (-90 bp / +67 bp) of wild type human *TGM1* promoter (Phillips et al. 2004), and the full length (2.2 Kb) human *TGM1* promoter. List of PCR primers is shown in Table 2.1. The resulting fragments were verified by sequencing and then cloned into a self-inactivating retroviral vector ((SIN γ -RV) HSC1-GiP, Addgene #58254) deprived of the internal human EF1 α promoter. The obtained constructs carry one of the three different promoters that guides the expression of a bicistronic cassette eGFP ires Puromycin. HSC1-GiP vector was used as a control for transfection and transduction in following experiments.

Generation of self-inactivating retroviral vector carrying the *TGM1* CDS. *TGM1* coding sequence (CDS) was synthesized by GeneArt (Invitrogen) and was cloned into a previously described replication-deficient self-inactivating retroviral vector (SIN γ -RV), under the control of the *TGM1* full promoter.

Table 2.1. List of primers used with specified name, sequence, and application

N°	Primer name	Sequence (5'-3')	Application
#1	TGM1 PromP1_Fw	ACCTAGTTAACGCTGAGTGTCTGCTCCCATG	PCR amplification
#2	TGM1 PromP1_R	TACAGGGCCGGCCTCCCAGAGAACCAGTAGGATG	PCR amplification
#3	TGM1 PromP2_Fw	ATTCTGGCCGGCCTGCTCCCTCCCTAGC	PCR amplification
#4	TGM1 PromP2_R	GATTTCCATGGTCAGGATGGATGGGAC	PCR amplification
#5	IVL PromP1_Fw	TTGGGTAAACAGCTTCTCCATGTGTCATG	PCR amplification
#6	IVL PromP1_R	GAAGGCCGGCCGGTCTTATGGGTTAGC	PCR amplification
#7	IVL PromP2_Fw	ATTAGGCCGGCCAGGAATAGTTGAGCTACCAG	PCR amplification
#8	IVL PromP2_R	GAAATCCATGGTGCTGAGCTGAGCAGGAG	PCR amplification
#9	TGM1 full Prom_Fw	AACTAGTTAACGCCAAGGCTTCAGTGTGTTG	PCR amplification
#10	TGM1 full Prom_R	ATTATGGCCGGCCGAGGTCTGGGGGCTTAGG	PCR amplification
#11	TGM1_Exon2_Fw	GATGGGCCACGTTCCGATG	PCR analysis for KO
#12	TGM1_Exon3_R	CGGGACAGGAGGAGGAGC	PCR analysis for KO
#13	TGM1_Intron3_Fw	CAGTGGCTCATACACATTGTG	PCR analysis for KO
#14	TGM1_Exon5_R	CTGCCGCCAATCCTCATGG	PCR analysis for KO

2.3 VIRAL PRODUCTION

Retrovirus vector preparation. Phoenix-AMPHO cells were cultured in DMEM supplemented with 10% heat-inactivated Fetal Bovine Serum, 1% Glutamine and 1% penicillin-streptomycin antibiotics (Thermo Fisher). High-quality DNA of each transfer vector was used for the transient transfection of Phoenix-AMPHO cells with branched PEI (Sigma), for retroviral production. The optimal DNA:PEI ratio was investigated for each plasmid in preliminary experiments before scaling up to a large-scale transfection. Transfection efficiencies were monitored by flow cytometry. Phoenix-AMPHO were transiently transfected with the retroviral plasmid - PEI mix for retrovirus vector preparations. Infectious retroviruses were harvested at 72 hours post-transfection, filtered through 0.45-µm pore cellulose acetate filters, and concentrated by ultracentrifugation (16 hours at 17,000g). The final viral pellets were resuspended in PBS – BSA 1% in ice, and aliquots of viral stocks were kept at –80 °C until further use. The viral titer was assessed on Hela cells by flow cytometry, or on human keratinocytes by immunofluorescence, and the typical viral titer was $1-2 \times 10^6$ UI/ml. Primary keratinocytes or HaCaT cells were infected with the retroviral stock at various multiplicities of infection (MOI) with Vectofusin-1 (Miltenyi Biotec) or RetroNectin® (Takara) addition (see Materials and Methods).

2.4 FLOW CYTOMETRY ANALYSIS

The proportion of green fluorescent protein (GFP)-positive and GFP intensity in Phoenix-AMPHO and Hela cells in order to determine the transfection efficiency and the viral titer, respectively, were monitored by flow cytometry (BD FACSCanto II), and data were collected using BD FACSDiva Software v6.1.3.

2.5 ANALYSIS OF HACAT CELLS TRANSDUCED WITH SINy -RVs

Generation of genetically modified HaCaT cell cultures. HaCaT cells were plated (3×10^3 cells per cm^2) in standard culture medium. The day before transduction, 4 wells of a 24-well non-treated cell culture plate were coated with a RetroNectin® Recombinant Human Fibronectin Fragment (Takara) solution (20 $\mu\text{g}/\text{ml}$ in DPBS (Gibco)) at 10 μg per cm^2 . The plate was then sealed with Parafilm® and incubated overnight at 4°C. In the morning, the RetroNectin® was removed and 0.5 ml of PBS-BSA 1% was added in each well. The plate was incubated for 30 min at room temperature (RT) and washed once with PBS. The SINy-RV-eGFP supernatants at MOI 5 were prepared in a final volume of 250 μl of medium and added to each well. Plates were incubated for 4h at 37 °C to allow virus precipitation. Once the incubation was completed, viral supernatants were discarded, and HaCaT cells were plated (1×10^4 cells per cm^2) in 250 μl of medium onto viral-coated wells. Cells were incubated at 37°C for 4h and, after that, 750 μl of fresh medium was added per well. 80% confluent HaCaT cells were expanded by plating all retrieved cells in a larger support. The samples were labeled as follows: *TGM1*min (HaCaT cells transduced with SINy-RV-eGFP with an internal *TGM1* minimal promoter), *TGM1*full (HaCaT cells transduced with SINy-RV-eGFP with an internal *TGM1* full promoter), IVLmin (HaCaT cells transduced with SINy-RV-eGFP with an internal IVL minimal promoter). Flow-cytometry analysis was performed (see Materials and Methods) in order to determine the percentage of transduced cells. Each condition of SINy-RV-eGFP transduced HaCaT cells was serially diluted and plated in 96-well plates (1 cell per well) for positive clone isolation. After 7 days of cultivation, the 96-well plates were checked under an inverted fluorescent microscope to select positive clones. eGFP⁺ clones were picked and expanded in serial passages. HaCaT cultures of each condition were frozen in liquid nitrogen (1×10^6 cells per vial) for further analysis.

Evaluation of eGFP fluorescent emission in HaCaT cells transduced with SINy -RVs.

Transduced HaCaT cells were thawed and cultured in Low Calcium conditions (LC) with 0.03 mM Ca^{2+} for at least 3 weeks, in order to induce a basal-like state which is characteristic of undifferentiated cells. During LC conditions HaCaT cells were monitored for morphology, protein, and RNA expression. After 3 weeks HaCaT cells were trypsinized

(Trypsin 2.5 % in HBSS w/o Phenol Red w/o Calcium w/o Magnesium, Euroclone) at 37°C for 15 min, and plated (2 wells for each condition) in a 6-well cell culture plate (1×10^4 cells per cm^2), in LC medium. The samples were labeled as follows: *TGM1min*, *TGM1full*, and *IVLmin*. Non transduced HaCaT cells were used as negative control. Three days after, one well of each condition was switched from LC medium to HC medium with 2.8 mM Ca^{2+} and the eGFP fluorescent emission was evaluated with a Cell Observer microscope (Axio Observer. Z1) using a 10x objective. An incubator was used to control temperature and CO_2 . Fluorescent and bright field micrographs of the samples were acquired every 24 hours for seven days.

Evaluation of eGFP expression in HaCaT cells transduced with SINy -RVs.

Undifferentiated transduced HaCaT cells were exposed to HC medium with 2.8 mM Ca^{2+} to induce differentiation. Cells were thawed and cultured for 24 h in LC (0.03 mM Ca^{2+}) prior to exposure to HC (2.8 mM Ca^{2+}). The medium was changed every 2 days. Decreasing numbers of cells are seeded for plates to be harvested on subsequent days to compensate for continued cell proliferation occurring during the differentiation process. Samples were harvested at time 0 (in Low Calcium), and at 8 h, 24 h, 48 h, 4 d, and 6 d, after calcium addition.

Provirus vector copy number (VCN). TaqMan PCR analysis was performed with TaqMan Universal PCR Master Mix and EGFP and genomic GAPDH probes. Reactions were performed with ABI Prism 7900 Sequence Detection System (Applied Biosystems), using 10 ng genomic DNA extracted from transduced HaCaT cells (see Materials and Methods). The relative quantity that relates to the PCR signal of the target provirus was normalized to the level of GAPDH (internal control gene) in the same genomic DNA by using the $2^{-\Delta\text{Ct}}$ quantification.

2.6 ANALYSIS OF HUMAN PRIMARY KERATINOCYTES TRANSDUCED WITH SINy-RVs

Generation of genetically modified human primary keratinocytes. Keratinocytes cell culture (K82) was thawed and plated (1.6×10^4 cells per cm^2) onto lethally irradiated 3T3-J2 cells (3×10^4 cells per cm^2) in KNO medium until sub-confluence was reached. Sub-confluent keratinocytes (K82) were trypsinized and diluted at 8×10^4 cells / μl . The three SINy-RV-eGFP vectors at MOI 1.5 (adjusted for 3T3-J2 cell that will seize part of the viral supernatant) were prepared in a final volume of 125 μl of KC w/o FBS w/o penicillin-streptomycin each. 5 μg / well of Vectofusin-1 solution was diluted in KC w/o FBS w/o penicillin-streptomycin, considering a final volume of 125 μl per well. Diluted viral

supernatants and diluted Vectofusin-1 solutions were mixed together and then added to the cell suspensions (2×10^4 cells / well). Each condition was plated on a 24-well cell culture plate onto lethally irradiated 3T3-J2 cells (3×10^4 cells per cm^2) and incubated at 36.5°C for 4h. After that, 500 μl of fresh KC 2x FBS medium was added per well. Three days after transduction, keratinocytes were trypsinized and plated onto lethally irradiated 3T3-J2 feeder-layer cells (3×10^4 cells per cm^2) to allow cell expansion. Two passages after transduction, keratinocytes were exposed to 2 μg / ml of puromycin (Sigma) for 72 hours for the selection of puromycin-resistant cells. Non transduced keratinocytes were used as a negative control of puromycin selection. Sub-confluent transduced keratinocytes cell cultures were trypsinized and frozen in liquid nitrogen (1×10^6 cells per vial) for further analyses.

Evaluation of eGFP expression in keratinocytes transduced with SINy -RVs. Sub-confluent transduced keratinocytes cell cultures were trypsinized and plated (3×10^4 cells per cm^2) in 6-well cell culture plates onto lethally irradiated 3T3-J2 cells (3×10^4 cells per cm^2) to induce differentiation in over-confluent conditions. Samples were feeder-depleted and harvested at 3 d, 7 d, 10 d, and 14 d of culture.

2.7 GENERATION OF 3D HUMAN SKIN EQUIVALENTS

Organotypic cultures preparation. 3T3-J2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% irradiated calf serum, glutamine (4 mM) and penicillin–streptomycin (50 IU/ml). 3T3-J2 cultures were used between passages 5 and 12. Alternatively, human fibroblasts have also been used. Fibroblast-populated collagen gels were prepared using collagen type I from rat tail (Corning) at a final concentration of 5 mg per ml. Collagen gels were prepared by mixing on ice one volume of 10X Ham's F12 nutrient mixture with one volume of 10X Reconstitution Buffer, type 1 collagen to a final concentration of 5 mg per ml, and 3T3-J2 cells (2×10^5 cells per ml) resuspended in FBS to reach the final volume. Each well of a 24-well plate was filled with 0.7 ml of the collagen and fibroblasts matrix, and the plate was incubated at 37°C and 5% CO_2 , to allow the gels to solidify. After 1 hour, 1 ml of fibroblasts culture medium was added per well. The following day, the collagen-fibroblast matrixes were seeded with primary human keratinocytes (1×10^5 cells per well) onto lethally irradiated 3T3-J2 cells (5×10^4 cells per well) in keratinocytes medium. After 5 days in submerged culture, the media was carefully removed, and the collagen gels were gently moved in millicell® cell culture inserts of 30-mm diameter with 0.4 μm pore size (Merk) and were further cultured for 20-24 days in air-liquid interface (ALI) condition to induce epidermal differentiation.

Cryopreservation and sectioning. 3D human skin equivalents were dehydrated in 30% sucrose at RT for thirty minutes and then passed to sucrose 2M for other thirty minutes. 3D skin equivalents were embedded with optimal cutting temperature freezing media (OCT) (Killik, Bio-Optica) and stored at -80°C. 5 µm sections of embedded skin equivalents were obtained with a histological cryomicrotome (Leica CM1850 UV).

2.8 RNA EXTRACTION AND QUANTITATIVE REAL-TIME PCR

Total RNA was extracted from primary human keratinocytes after feeder-layer depletion, or from HaCaT cells. Total RNA was extracted using the PureLink RNA Mini Kit (Thermo Fisher) according to the manufacturer's instructions. Complementary DNA was generated using the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher). Real-time qPCR analyses were carried out on triplicate samplings of retrotranscribed cDNAs with Taqman Universal PCR Master mix on 7900H Real-Time PCR System (Thermo Fisher). Expression levels are given relative to human GAPDH. List of TaqMan probes (Thermo Fisher) is provided in Table 2.2. Data were analyzed using the 2- Δ Ct quantification and visualized with Prism 8.

Table 2.2. List of TaqMan® probes used for qRT-PCR and catalog number

TaqMan® probes	Company	Catalog number
eGFP	Thermo Fisher	Mr00660654_cn
GAPDH	Thermo Fisher	4352665
Genomic GAPDH	Thermo Fisher	Hs03929097_g1
K10	Thermo Fisher	Hs00166289_m1
TGM1 Exon 14-15	Thermo Fisher	Hs00165929_m1
TGM1 Exon 3-4	Thermo Fisher	Hs01070310_m1

2.9 PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

Samples of human primary keratinocytes destined to Western analysis were depleted of feeder layer. Cells were lysed in RIPA buffer (Sigma Aldrich) supplemented with phosphatase and Protease Inhibitor Cocktail (Thermo Fisher) for 30' on ice. BCA kits (Pierce) were used to quantify the total protein amount. The same number of proteins was loaded in 4–12% NuPAGE Bis-Tris Gels or 10% NuPage Tris-Acetate Gels (Thermo Fisher) at 100 V for 2 h and transferred 100 V at 4 °C for 2 h onto nitrocellulose membrane (Millipore). Membranes were treated with EveryBlot blocking buffer (Bio-Rad) for 5 minutes with agitation. Primary antibodies were diluted in blocking buffer as indicated in Table 2.3 and added overnight at 4 °C to the membranes. Secondary antibodies were diluted in

Blocking buffer as indicated in Table 2.3 and added to the corresponding membranes for 1 h at room temperature. Signal was visualized with Clarity Western ECL substrate (Bio-Rad) using ChemiDoc (Bio-Rad). Bio-Rad's Image Lab software 6.0 was used for band densitometry. Gray background on the images was homogeneously added for graphical purposes.

Table 2.3. List of antibodies, source, and concentration

Antibody information				Dilution/amount	
Antibody	Company	Catalog number	Description	WB	IF
anti-TGM1	Invitrogen	PA5-59088	Rabbit polyclonal	1/1000 - 1/4000	
anti-TGM1	Merk	HPA040171	Rabbit polyclonal		1/2000
anti-Cytokeratin 14	BioLegend	905301	Rabbit polyclonal		1/150,000
anti-Collagen 17A1	Genetex	GTX54647	Mouse monoclonal		1/100
anti-SPINK5	Merk	HPA011351	Rabbit polyclonal		1/2000
anti-p63 alfa	Di Iorio et al., 2005	N/A	Rabbit monoclonal		1/10,000
anti-human GAPDH	Merk	ZRB374 - clone 10B13	Rabbit monoclonal	1/1000	
anti-GAPDH	Abcam	ab8245	Mouse monoclonal	1/10,000	
anti-Involucrin	Leica	NCL-INV	Mouse monoclonal	1/2000	1/1000
anti-Cytokeratin 10	BioLegend	905403	Rabbit polyclonal	1/500	1/1000
anti-GFP	Santa Cruz Biotechnology	sc-9996	Mouse monoclonal	1/250	
anti-GFP	Merk	AB10145	Rabbit polyclonal	1/1000	1/500
anti-Vimentin	BioLegend	699302	Rat		1/1000
Alexa Fluor 568 anti-rat	Thermo Fisher Scientific	A11077	secondary antibody		1/1000
Donkey anti-rabbit IgG HRP	Santa Cruz Biotechnology	sc-2313	secondary antibody	1/2000	
Donkey anti-mouse IgG HRP	Santa Cruz Biotechnology	sc-2314	secondary antibody	1/10,000 - 1/20,000	
Donkey anti-Mouse IgG (H+L) Alexa Fluor 568	Thermo Fisher Scientific	A10037	secondary antibody		1/2000 - 1/1000
Donkey anti-Rabbit IgG (H+L) Alexa Fluor 488	Thermo Fisher Scientific	A21206	secondary antibody		1/2000 - 1/1000

2.10 HISTOLOGY AND IMMUNOFLUORESCENT STAINING

For immunofluorescence analyses of in vitro cultured keratinocytes, cells were plated at 1000/wells onto glass coverslips and grown as previously described. Cells were fixed with PFA 3% for 10 min at room temperature, carefully washed with 1X PBS and permeabilized in PBS/Triton 0.3% for 15 min. Blocking solution (FBS 5% BSA 2% in PBS/Triton 0.1%) was added for 1 h at RT. Primary antibodies were diluted in Blocking solution as described in Table 2.3 and added to the samples overnight at 4 °C. Secondary antibodies were diluted in Blocking solution as described in Table 2.3 and added to the samples for 1 h at RT. Cell

nuclei were stained with DAPI. Dako Mounting medium was used to mount coverslips. Zeiss confocal microscope LSM510meta with a Zeiss EC Plan-Neofluar 40X/1.3 oil immersion objective was used to visualize fluorescent signals.

Immunofluorescence staining of 3D skin equivalents was performed on 7- μ m cryosections. In brief sections were fixed in PFA 3% or methanol, permeabilized for 15 min with PBS/Triton 0.3%, and blocked for 30 min at 37 °C with Blocking solution (with BSA 5% in PBS). The primary antibody, diluted in Blocking solution, was added to skin sections for 1 h at 37 °C. Sections were washed three times in Wash solution (PBS Triton 0,1%) and incubated with secondary antibody diluted in Blocking solution as described in Table 2.3 for 1 h at r.t. Cell nuclei were stained with DAPI for 3 min at room temperature. Glasses were then mounted with Dako Mounting medium and fluorescent signals were monitored under a Zeiss confocal microscope LSM510meta with a Zeiss EC Plan-Neofluar 40X/1.3 oil immersion objective. The antibodies used for immunofluorescence are described in Table 2.3.

Hematoxylin and eosin staining was performed on 7- μ m cryosections of 3D skin equivalents. Bright field color images were taken with a Zeiss microscope Axio Imager M2 with an EC Plan-Neofluar 40X/0.75 M27 air objective.

2.11 GENERATION OF KNOCK OUT *TGM1* HUMAN KERATINOCYTES

Guide RNAs design. gRNA CRISPR guides were designed targeting *TGM1* E2 (gRNA1, 5' AAATGCGGCAGATGACGACT1 3' and gRNA2, 5' AAATGCGGCAGATGACGACG 3', which differ for one nucleotide (underlined) allowing to target both *TGM1* alleles) and *TGM1* E4 (gRNA3, 5' GGATGTAGATCTCATTGCGG 3') sequences. gRNAs were checked for the minimal off-target activity predicted by an online tool (<http://www.rgenome.net/cas-offinder/>).

Ribonucleoprotein (RNP) complex formation and Nucleofection. The three synthetic guide RNAs (Invitrogen) were mixed with the Cas9 protein (Alt-R S.p. Cas9 Nuclease V3, IDT) in a 0.3:0.3:0.3:1 ratio and incubated at room temperature for 10-20 minutes, forming the RNP complex. 5×10^5 keratinocytes were resuspended in 100ml primary cell nucleofection solution (P3 Primary Cell 4D-Nucleofector Kit, Lonza), mixed with the RNP complex solution and 4mM Cas9 electroporation enhancer (IDT). Cells (K81 P3) were electroporated using a 4D-Nucleofector (Lonza) using the program DS-138. After nucleofection, keratinocytes were plated (6×10^3 cells/cm²) on lethally irradiated 3T3-J2 cells. To ensure a total biallelic KO of *TGM1* sequence, sub-confluent keratinocytes (K81 P4) were electroporated again and plated on lethally irradiated 3T3-J2 cells, as previously

described. The samples were labeled as follows: Δ TG1.1 (primary keratinocytes electroporated with the RNP complex), Δ TG1.2 (primary keratinocytes electroporated with the RNP complex twice).

Genomic analysis of TGM1 KO Keratinocytes. Genomic DNA (100ng) isolated from TGM1-KO and non-treated keratinocytes (see Materials and Methods) was used for the screening of the TGM1 sequence through PCR amplification with a GoTaq® polymerase (Promega). Two couple of primers (#11 - #12; #13 - #14, Table 2.1) were used to investigate the genomic sequence flanking both edited exons (e.g, E2 and E4, respectively). Moreover, PCR fragments spanning either the Cas9 target sites, were generated to evaluate the excision of the entire genomic region of above 1.7 Kb between E2 and E4, with primers #11 and #14 (list of primers and PCR programs are reported in Table 2.1). PCR products were analyzed in 1% agarose gel. The molecular weight markers were 100bp DNA Ladder (Invitrogen) or 1 Kb plus DNA Ladder (Invitrogen). Bio-Rad's Image Lab software 6.0 was used for PCR band densitometry.

TGM1 mRNA expression in KO Keratinocytes. Sub-confluent KO keratinocytes cells were trypsinized and plated (3×10^4 cells per cm^2) in 6-well cell culture plates onto lethally irradiated 3T3-J2 cells (3×10^4 cells per cm^2) to induce differentiation in over-confluent conditions. Samples were feeder-depleted and harvested at 3 d, 7 d, 10 d, and 14 d of culture. Total RNA was extracted, and complementary DNA was generated as previously described (see Materials and Methods). Real-time qPCR analyses were carried out to determine TGM1 expression levels during differentiation. Data were analyzed with RQ Manager Software 1.2.2 and visualized with Prism 8.

Immunofluorescence analysis of TGM1 KO Keratinocytes. Sub-confluent KO keratinocytes cell cultures were trypsinized and plated (3×10^3 cells per well) onto treated glass coverslips and grown as previously described. Cells were fixed with PFA 3% and stained with TG1 antibody (see Table 3) for immunofluorescence analysis.

Mutation detection by decomposition (TIDE). TGM1 knock out efficiency was performed with the TIDE web tool. Amplified genomic regions flanking edited exons, from DNA of the control and experimental samples (100ng), were generated with primers (#11 - #12; #13 - #14) and were subjected to conventional Sanger sequencing according to the protocol of Eurofins Genomics. The resulting sequence trace files were uploaded on TIDE web tool with the guide RNAs sequences as input. Obtained data were visualized with Prism 8.

2.12 TG1 *in situ* ACTIVITY ASSAY

TG1 *in situ* activity was performed on unfixed cryosections (5- μ m) as reported in literature (Takeda et al. 2018), with minor modifications. Assay was based on the reaction of transglutaminase with its substrate cadaverine in a reaction buffer containing calcium. The 3D skin equivalent cryosections were air-dried for 10 min at RT and then blocked with 1% bovine serum albumin in phosphate buffered saline for 30 min at RT. After, the sections were incubated in 100 mmol/L Tris-HCl pH 7.4, 5 mmol/L CaCl₂ and 0.1 mmol/L Alexa Fluor™ 594 Cadaverine (A30678; Thermo Fisher Scientific) for 90 min at RT. Cell nuclei were stained with DAPI for 3 min at RT. Glasses were then mounted with Dako Mounting medium and fluorescent images were obtained using a Zeiss confocal microscope LSM510meta with a Zeiss EC Plan-Neofluar 20X/0.50 M27 air objective.

2.13 TG1 *in vitro* ACTIVITY ASSAY

TG1 *in vitro* activity assay was performed on 50 μ g of proteins obtained from biological samples as previously described (see Materials and Methods), using the Transglutaminase assay kit (CS1070; Merk) according to the manufacturer's instruction. The assay is based on transglutaminase catalysis of a covalent bond formation between the free amine group of Poly L-Lysine, which is covalently attached to the 96-well plate surface, and the γ -carboxamide group of biotin substrate present in the assay buffer. This reaction immobilizes biotin to the plate surface. The amount of immobilized biotin, which is proportional to the amount of active transglutaminase in the sample, is determined using streptavidin-peroxidase and TMB substrate. Briefly, 50 μ g of protein extracts were added to each well. 2 milliunits/ml of Transglutaminase enzyme from guinea pig liver were used as positive control. 50 μ l of Assay Mixture were added to each well and the plate was incubated for 15-30 min at RT. Streptavidin-Peroxidase and TMB substrate were added to each well and the absorption was read at 450 nm with a plate reading spectrophotometer (Glomax; Promega).

2.14 ISOLATION OF CROSS-LINKED ENVELOPES

For spontaneous envelope formation, human primary keratinocytes were seeded onto a millicell® cell culture insert of 12-mm diameter with 0.4 μ m pore size (Merk) for 5 days in submerged culture, and further cultured for 20-24 days in air-liquid interface (ALI) condition to induce epidermal differentiation. After, the insert was carefully removed with a tweezer and submerged in a buffer-solution consisting of in 1 M Tris-HCl (pH 8.0), 2% 2-mercaptoethanol and 2% SDS. The suspension was heated to 100°C for 10 min and vortex-

mixed for 10 min. Envelope's suspension was assessed by phase contrast microscopy (Jeon et al. 1998).

2.15 STATISTICAL ANALYSES

Data are presented as the mean $\pm$ SD. For real-time semi-quantitative reverse-transcription-PCR experiments, significance was calculated from Δ Ct values with ANOVA test. A value of $P < 0.001$ was considered statistically significant. In these experiments, two types of significance were analyzed: significance between cells transduced with the same promoter in HC medium (HaCaT cells) or in over-confluent conditions (K82), over time (* $P < 0.0001$), and significance between wild-type human primary keratinocytes (K81) and *TGM1* KO keratinocytes (Δ TG1.1), (* $P < 0.0001$).

Aim

Given the successful clinical outcome of *LAMB3*-gene therapy for junctional epidermolysis bullosa provided by our group (Mavilio et al. 2006; Bauer et al. 2017; Hirsch et al. 2017) the purpose of this project is to develop a similar combined cell and gene therapy approach aimed at correcting the *TGM1* mutations underlying LI.

Specific aim of this thesis: (i) development of a self-inactivating retroviral vector (SINy-RV) with different promoters to grant the physiological expression of the *TGM1* gene; (ii) development of a LI cellular model using the CRISPR/Cas9 technology to knock out *TGM1* in primary human keratinocytes; (iii) to prove the feasibility of SINy-RV-*TGM1* gene therapy in *TGM1*-KO cellular model; (iv) to generate 3D skin equivalents to analyze the physiological expression of TG1 in the stratified epidermis and demonstrate TG1 protein restoration, as well as its enzymatic activity post gene correction. These findings will serve as a blueprint for the development of combined cell and gene therapy for *TGM1*-dependent LI and pave the way for a faster application of the proposed approach in Phase I/II clinical trials for the treatment of skin defected in LI patients.

3. RESULTS

3.1 Promoter comparison for physiological *TGM1* expression in primary differentiated keratinocytes

Construction of self-inactivating retroviral vectors

To confer tissue- and stratum-specific expression to TG1 in the upper layers of the epidermis, we compared the activity of three promoters in driving the expression of eGFP in primary human keratinocytes: the 637-bp human Involucrin minimal promoter (IVL-min), the 367-bp human *TGM1* minimal promoter (TGM1-min) and the 2.2-Kb human *TGM1* full length promoter (TGM1-full). The structure of the SINy-RVs carrying the eGFP coding sequence under the control of such promoters is shown in **Figure 3.1**.

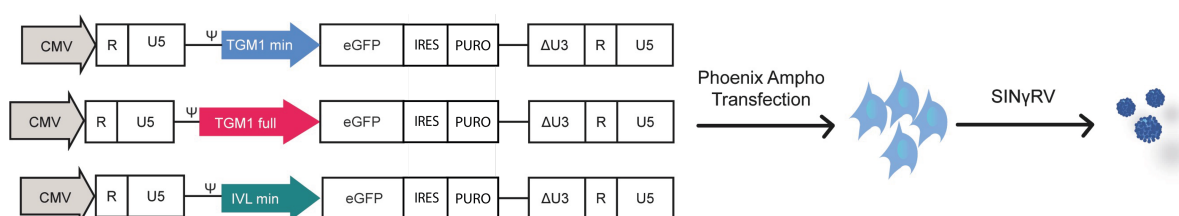


Figure 3.1. Self-inactivating retroviral vectors. Schematic representation of the developed self-inactivating retroviral vectors (SINy-RVs) carrying the three different promoters driving the expression of the bicistronic gene encoding enhanced green fluorescent protein (eGFP) and puromycin resistance.

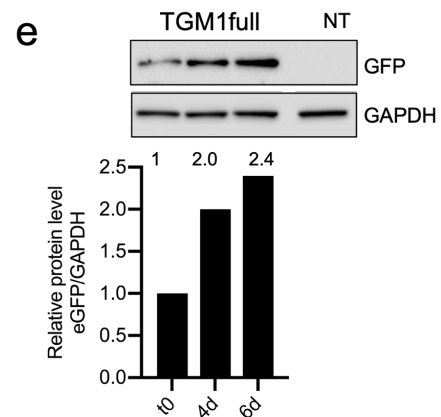
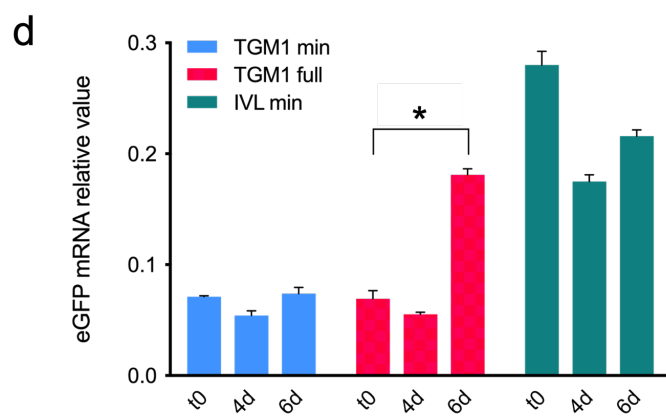
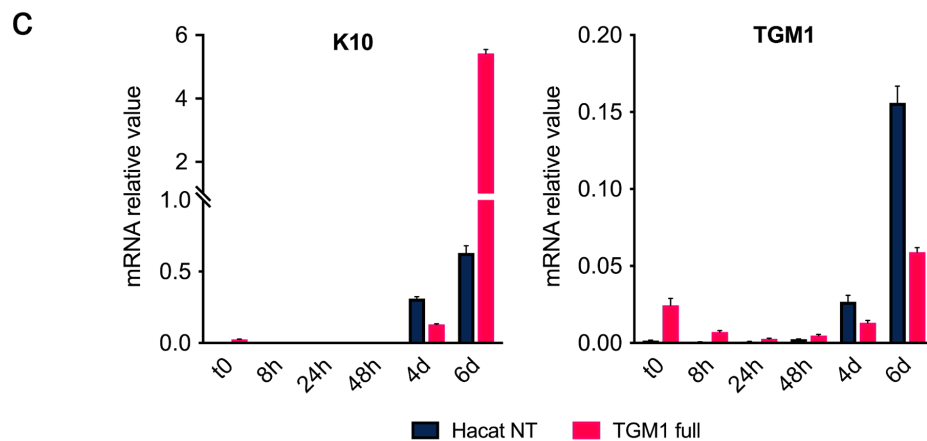
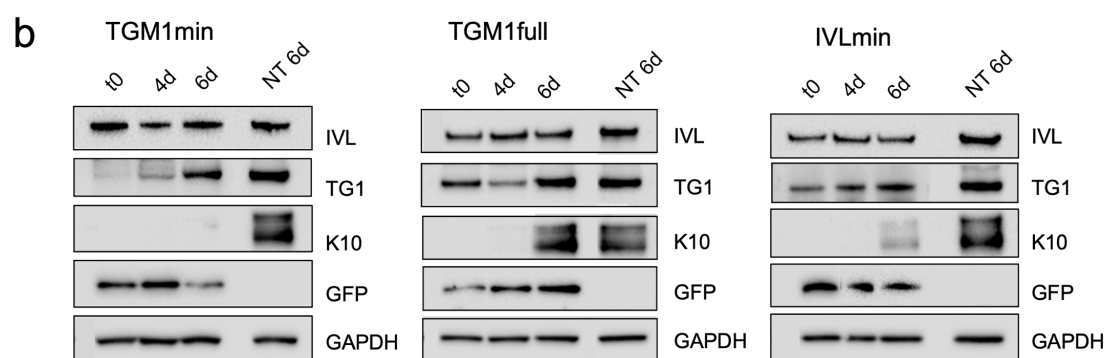
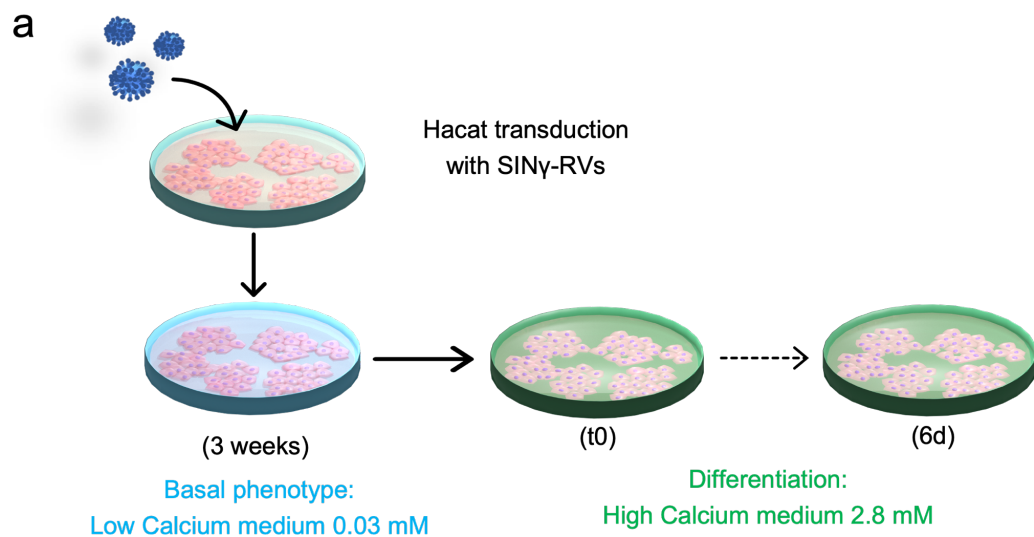
Differentiation stimuli induces the expression of eGFP in HaCaT cell cultures transduced with self-inactivating retroviral vectors.

To investigate the differentiation-related eGFP expression driven by the different promoters, HaCaT cells were transduced with the SINy-RV-eGFP vectors (**Figure 3.1**) at MOI 5. Transduction efficiency ranged between 50 and 65%. Comparison of absolute levels of eGFP expression (**Figure 3.2. d**) is not meaningful, since HaCaT cells transduced with the IVL minimal promoter have an average vector copy number per cell 4 times higher than that of the other promoters (data not shown). We thus investigated how the different promoters were regulated by the execution of the keratinocyte terminal differentiation program.

SINy-RV-eGFP transduced HaCaT cells were serially diluted to isolate a homogeneous eGFP⁺ cell population, which were then cultured in keratinocytes serum free medium (KSFM) in low calcium (LC) conditions (0.03 mM Ca²⁺) for at least 3 weeks and then

switched to high calcium (HC) conditions (2.8 mM Ca²⁺) to induce differentiation (**Figure 3.2. a**). Differentiation of HaCaT cells was evaluated with western blot and qRT-PCR analyses, in both non-transduced (NT) and transduced cells (**Figure 3.2. b** and **c**, respectively), at different time points in HC medium. The first time point (t₀) can be considered the basal level. The expression of epidermal differentiation specific markers such as K10, TG1 and involucrin (IVL) increased in HC conditions as compared to the basal level (time zero, t₀), confirming proper differentiation stimuli. We then analyzed the transcriptional activity of the different promoters following differentiation stimuli in this cellular context.

Strikingly, mRNA and eGFP protein expression unveiled that only the *TGM1* full promoter is properly regulated during keratinocyte differentiation (**Figure 3.2. b, d, and e**).



(Legend on next page)

Figure 3.2. Differentiation-related expression of eGFP in HaCaT cell cultures transduced with SIN γ -RVs. **a)** Graphic representation of the HaCaT differentiation model. **b)** Western blot analysis of the expression of eGFP and keratinocyte differentiation markers (K10, TG1, and IVL) in HaCaT cells transduced with *TGM1* minimal promoter-eGFP (*TGM1min*), *TGM1* full promoter-eGFP (*TGM1full*) or IVL minimal promoter-eGFP cassette (*IVLmin*), grown in LC (t0) and HC medium for 4 or 6 days (4d, 6d). Non transduced HaCaT cells (NT) at 6 days after calcium addition are loaded as a reference sample. **c)** K10 (left) and TGM1 (right) mRNA relative expression in non-transduced HaCaT cells (NT) and HaCaT cells transduced with *TGM1* full promoter-eGFP (*TGM1full*) grown in LC (t0) and HC medium for 8, 24, 48 hours, 4 or 6 days. Expression levels were normalized per GAPDH. **d)** qRT-PCR quantification of the mRNA relative value of eGFP in HaCaT cells transduced with SIN γ -RVs grown in LC (t0) and HC medium for 4 or 6 days (4d, 6d). Statistical analysis: ANOVA test; asterisk indicates $p < 0.0001$. **e)** Top: highlight of eGFP protein expression in HaCaT cells transduced with *TGM1* full promoter-eGFP cassette (*TGM1full*). Bottom: densitometric analysis of eGFP/GAPDH protein ratio in HaCaT cells transduced with *TGM1* full promoter grown in LC (t0) and HC medium for 4 or 6 days (4d, 6d).

A further analysis was carried out to validate the previously reported data. Transgenic HaCaT cells were grown in low calcium (0.03 mM Ca²⁺) for three weeks. Live-cell imaging analysis of eGFP immunofluorescent signal was then performed to investigate the timing of the promoter transcriptional activity following the induction of terminal differentiation by high calcium (2.8 mM Ca²⁺) medium, with was added to induce differentiation. As shown in **Figure 3.3. (a, b and c)**, eGFP was constitutively expressed in HaCaT cells transduced with *TGM1* min (**a**) and IVL min (**b**) promoters, while in HaCaT cells transduced with *TGM1* full promoter, the eGFP fluorescent signal started from day 5 and progressively increased during terminal differentiation (**c**), further confirming that only the full promoter is properly regulated.

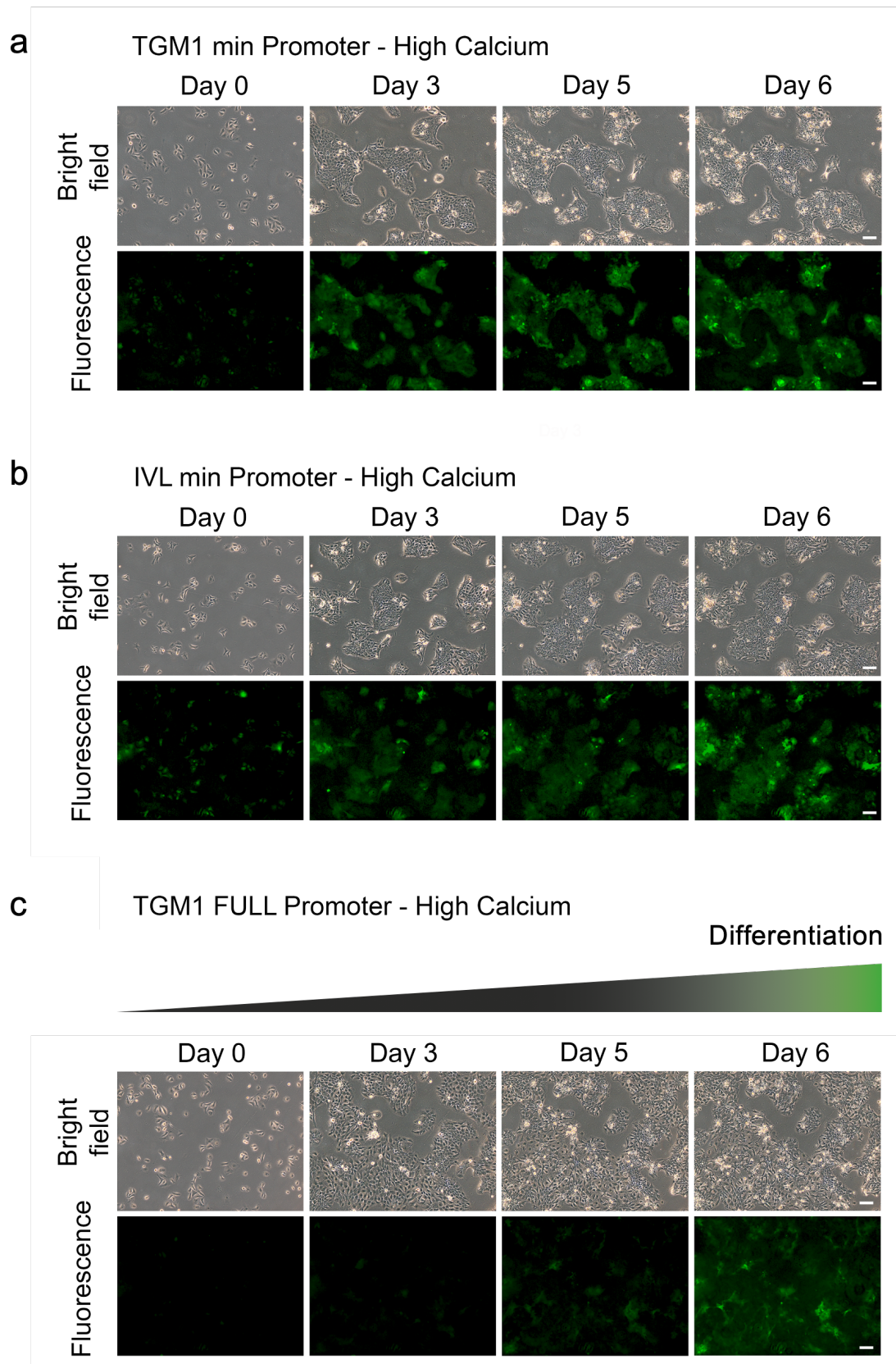


Figure 3.3. Live-cell imaging of HaCaT cells transduced with SINy-RVs. Representative bright-field and fluorescent micrographs of HaCaT cells transduced with **a)** *TGM1* minimal promoter-eGFP (*TGM1min*), **b)** *IVL* minimal promoter-eGFP (*IVLmin*), or **c)** *TGM1* full promoter-eGFP (*TGM1full*) cassette, grown in HC medium for 7 days. All images were acquired with the same magnification. Scale bars correspond to 50 μ m.

Primary human keratinocyte cultures

Data produced with HaCaT cells were then validated in primary cultures of human keratinocytes. In addition to calcium, other factors can directly act on differentiation of primary keratinocytes in vitro (Borowiec et al. 2013). For instance, keratinocyte confluence strongly induces terminal differentiation, as demonstrated by the rapid induction of the K10 and involucrin early differentiation markers (Poumay and Pittelkow 1995). The development of intimate cell-cell contacts associated with cell confluence serves as a crucial signal for keratinocytes to initiate the differentiation program (Pillai et al. 1990; Lee et al. 1998).

Therefore, the activation of the three different promoters was evaluated in growing, confluent and over-confluent transgenic human primary keratinocytes, as schematically shown in **Figure 3.4. a**. Primary keratinocytes (K82) were transduced with the SIN γ -RV-eGFP vectors (**Figure 3.1**) at MOI 2. For each condition keratinocytes were exposed to 2 μ g/ml Puromycin to isolate a homogeneous eGFP⁺ cell population. The expression of some differentiation markers was evaluated in non-transduced (K82 NT) and transduced cells, cultured for 3, 7, 10, and 14 days (**Figure 3.4. b**, and **c**). Western blot and qRT-PCR analyses showed a density-dependent expression of differentiation markers. Moreover, no significant differences were observed between non-transduced and transduced keratinocytes, demonstrating that the retroviral-mediated expression of eGFP and the following puromycin selection did not adversely influence keratinocytes differentiation. As previously investigated in HaCaT cells, we analyzed the differences in the reporter gene expression (eGFP) among the three different promoters following differentiation stimuli. As expected, the analysis of eGFP mRNA expression revealed that only the *TGM1* full promoter was properly regulated during keratinocyte differentiation (**Figure 3.5 a**). Indeed, the mRNA and protein expression of eGFP in keratinocytes transduced with *TGM1* full promoter progressively increased, in a density-dependent manner (**Figure 3.5 a**, and **c**), confirming that the activation of this promoter is properly regulated. Furthermore, the ratio of eGFP mRNA expression from day 3 to day 14 in transduced keratinocytes is very similar to that of *TGM1* mRNA expression of wild-type, non-transduced keratinocytes. Thus, we can assume that the exogenous *TGM1* full promoter has the same transcriptional activity as the endogenous one (**Figure 3.4. a** and **b**).

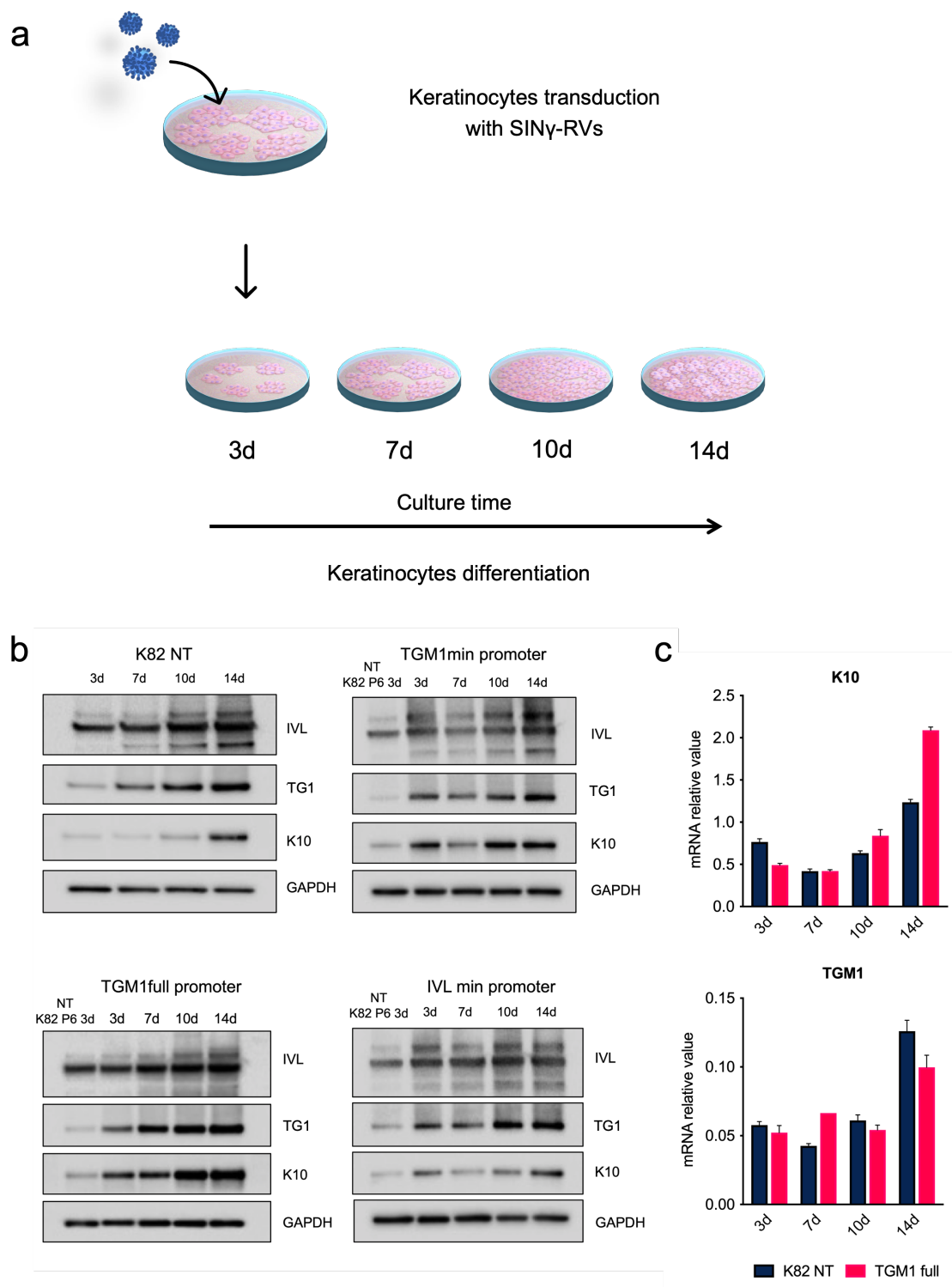


Figure 3.4. Differentiation of cultured human primary keratinocytes at increasing cell densities. **a)** Graphic representation of *in vitro* keratinocytes differentiation experiment. **b)** Western blot analysis of keratinocyte differentiation markers (K10, TG1, and IVL) in wild-type keratinocytes (NT, K82 P6) and keratinocytes transduced with *TGM1* minimal promoter-eGFP, *TGM1* full promoter-eGFP or IVL minimal promoter-eGFP cassette, grown in standard culture medium for 3, 7, 10 or 14 days (3d, 7d, 10d, 14d). **c)** K10 (top) and TGM1 (bottom) mRNA relative expression in non-transduced keratinocytes (K82 NT) and keratinocytes transduced with *TGM1* full promoter-eGFP (TGM1full) grown in standard culture medium for 3, 7, 10 or 14 days (3d, 7d, 10d, 14d). Expression levels were normalized per GAPDH.

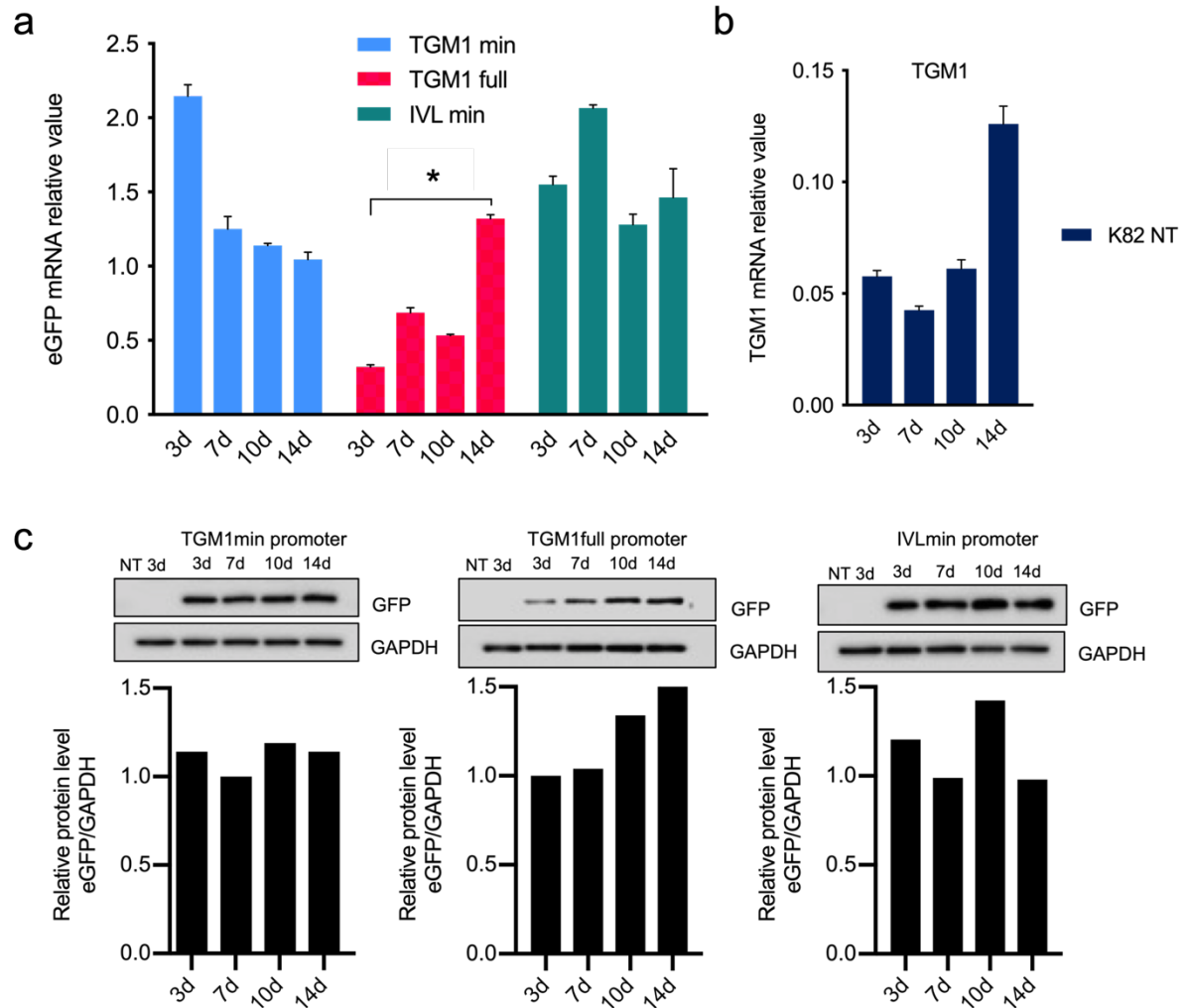
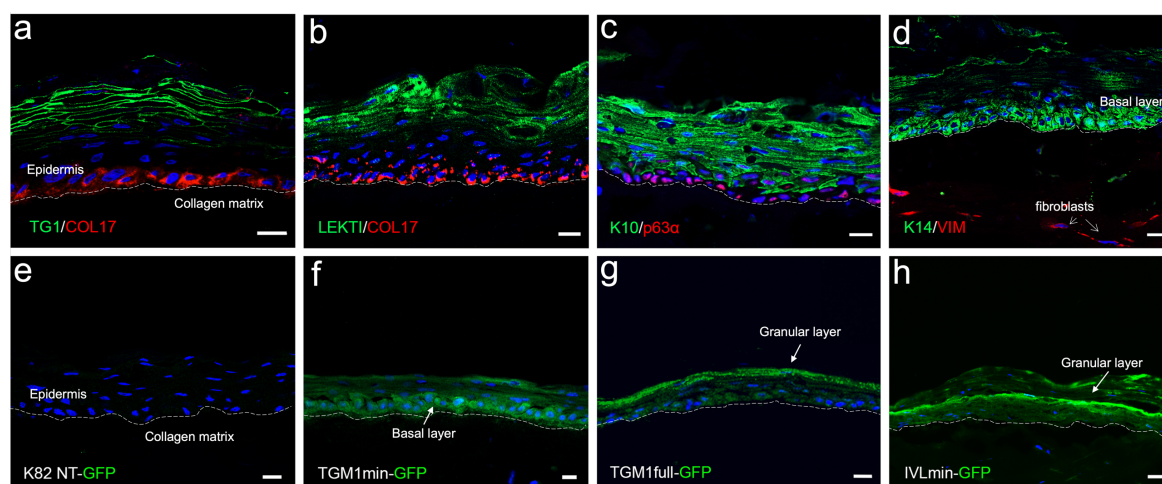


Figure 3.5. Differentiation-related expression of eGFP in human primary keratinocytes transduced with SINY-RVs. **a)** qRT-PCR quantification of the mRNA relative value of eGFP in keratinocytes transduced with *TGM1* minimal promoter-eGFP (TGM1min), *TGM1* full promoter-eGFP (TGM1full) or IVL minimal promoter-eGFP cassette (IVLmin), grown in standard culture medium for 3, 7, 10 or 14 days (3d, 7d, 10d, 14d). Expression levels were normalized per GAPDH. Statistical analysis: ANOVA test; asterisk indicates $p < 0.0001$. **b)** mRNA relative value of *TGM1* in non-transduced keratinocytes (K82 NT) under the previously described culture conditions. Expression levels were normalized per GAPDH. **c)** Top: eGFP protein expression in keratinocytes transduced with *TGM1* minimal promoter-eGFP (TGM1min), *TGM1* full promoter-eGFP (TGM1full) or IVL minimal promoter-eGFP cassette (IVLmin), grown in standard culture medium for 3, 7, 10 or 14 days. Bottom: densitometric analyses of eGFP/GAPDH protein ratio.

Three-dimensional cell culture techniques for developing skin equivalents

The 3D skin equivalent is a model for a robust and reproducible assay that is widely applicable to dermatological research, mimicking the morphology and microenvironment of normal human skin more accurately than 2D cell culture assays. Skin equivalents are designed to closely resemble native skin and can be developed from primary keratinocytes and fibroblasts and components of the extracellular matrix (ECM). This assay may substantiate the feasibility of the proposed therapeutic approach in a more *in vivo*-like context than traditional 2D cell cultures. Especially for the study of inherited disorders of cornification, the use of 3D skin equivalents for the generation of a fully differentiated epidermis is mandatory (Niehues et al. 2018).

To this end, non-transduced and transduced keratinocytes were seeded onto a collagen matrix containing living fibroblasts and cultured for 1 week in submerged culture and then for three weeks in air-liquid interface, allowing them to fully differentiate (see Materials and Methods). 3D cultures were fixed, sectioned and immunostained to examine morphology, differentiation markers as well as eGFP protein expression in a biomimetic and physiological environment. Differentiation markers, such as K10, TG1 and LEKTI were correctly localized and expressed in the upper layers of differentiated epidermis (**Figure 3.6. a, b, and c**), whilst the expression of K14, COL17 and p63 α was correctly restricted to the epidermal basal layer (**Figure 3.6. a, b, c, and d**). Moreover, the fibroblasts contained within the 3D skin equivalent clearly expressed vimentin (**Figure 3.6. d**), which indicates their viability and their constant production of secreted factors, critical for the maintenance of the 3D skin equivalent.



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Figure 3.6. Expression of epidermal markers and eGFP in organotypic 3D skin cultures. Representative immunofluorescent images of 7µm-thick cryosections of 3D human skin equivalents obtained with wild-type keratinocytes showing the expression of TG1 and COL17 (**a**), LEKTI and COL17 (**b**), K10 and p63α (**c**), K14 and VIM (**d**). Representative immunofluorescent images of 7µm-thick cryosections for the expression of eGFP in 3D human skin equivalents obtained with wild-type keratinocytes (K82 NT, **e**), and keratinocytes transduced with *TGM1* minimal promoter-eGFP (*TGM1*min, **f**), *TGM1* full promoter-eGFP (*TGM1*full, **g**) or IVL minimal promoter-eGFP cassette (*IVL*min, **h**). Abbreviations: TG1, Transglutaminase 1; LEKTI, Lympho-epithelial Kazal-type-related inhibitor; COL17, Collagen XVII; K10, Cytokeratin-10; K14, Cytokeratin-14; VIM, Vimentin. DAPI (blue) stains nuclei. White dotted line marks the epidermal-dermal junction. Scale bars, 20 µm.

Given the successful recapitulation of epidermal stratification in 3D human skin equivalents *in vitro*, we explored the ability of the different promoters to restrict the expression of eGFP to the layers that physiologically express TG1 and IVL *in vivo*. Organotypic cultures were constructed with keratinocytes transduced with retroviral vectors in which eGFP expression was driven by *TGM1* minimal, *TGM1* full or involucrin minimal promoter. eGFP expression (green) was observed in all layers of cultures seeded with *TGM1* min-eGFP-transduced keratinocytes indicating that this promoter has a pan-epithelial expression (**Figure 3.6. f**). In contrast, *TGM1* full and IVL min promoters properly directed eGFP expression to the granular layer of the cultures (**Figure 3.6. g**, and **h**). These data demonstrate that retroviral vectors containing both *TGM1* full and IVL min promoters were able to direct the expression of a reporter gene to the upper differentiated layers of the epidermis.

3.2 Generation of Δ TGM1 human primary keratinocytes

Before asking for skin biopsies of LI patients, we decided to knock out *TGM1* in primary human keratinocytes, hence establish a reliable cellular model able to recapitulate the LI pathogenic phenotype.

To date, the most efficient tool able to ensure a complete gene knockout is the CRISPR-Cas9 system (Taylor and Woodcock 2015; Schuster et al. 2019). Therefore, we performed a *TGM1* gene knockout (KO) on human primary keratinocytes from healthy donors. Three different gRNAs were designed to target both *TGM1* alleles (**Figure 3.7. a and b**). gRNA1 and gRNA2, that were designed to target the Exon 2 (E2) of the *TGM1* gene, differ for one nucleotide closed to the PAM sequence, which makes them allele specific. While the gRNA3 was designed to target the Exon 4 (E4). E2 and E4 were chosen as KO target sites for their crucial role in determining the protein function. Indeed, the E2 encodes for an N-terminal sequence unique to TGase 1 that is required for membrane anchoring, and the E4 encodes for a part of the catalytic core domain of the TG1 enzyme.

Human primary keratinocytes (K81) were electroporated with the ribonucleoprotein (RNP) complexes containing the three different gRNAs, in two steps. The first electroporation generated Δ TG1.1 keratinocytes, while Δ TG1.2 keratinocytes were obtained after one culture passage and another electroporation step, in order to increase the editing efficiency. The corresponding edited sequence in both exons, were assessed by PCR amplification of fragments spanning the target sites (**Figure 3.7. c**). Using specific primers designed on target sites, the PCR amplification did not generate an amplicon in both Δ TG1.1 and Δ TG1.2 keratinocytes, demonstrating the gene ablation. Moreover, the PCR amplification performed with primers spanning both the Cas9 target sites demonstrated the excision of the entire genomic region (1.7 Kb) between E2 and E4 in 67% and 61% of cases, for Δ TG1.1 and Δ TG1.2 keratinocytes, respectively (**Figure 3.7. d**). This result is shown by the presence of smaller bands with a size congruent with the distance between the annealing sites of the PCR primer used. However, to further validate the efficiency of *TGM1* ablation, the PCR amplicons of Exon 2 and Exon 4 were sequenced and analyzed using the TIDE web tool (<http://tide.nki.nl>) (**Figure 3.7. e**), as compared to the wild-type sequences. The overall editing efficiencies, reaching 100%, is indicated by the absence of non-edited sequences (point 0 of the graphs) and the presence of a broad spectrum of insertions and deletions in the analyzed samples.

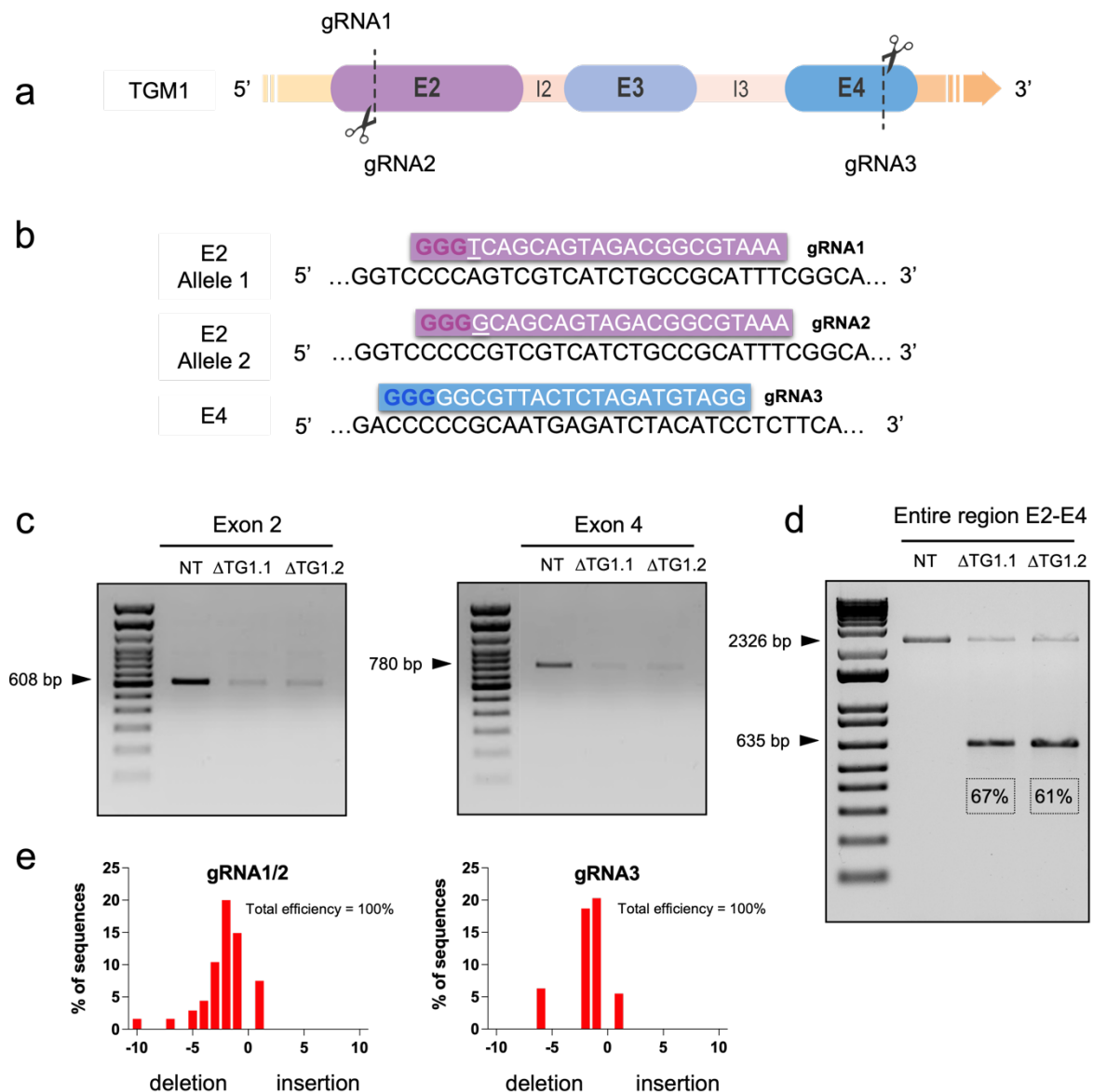


Figure 3.7. TGM1 knockout in human keratinocytes with the CRISPR-Cas9 system.

a, b) *TGM1*- knockout strategy. Three gRNA guides were designed to target *TGM1* Exon 2 (gRNA1 and gRNA2, which differ for one nucleotide (underlined) allowing to target both alleles) and *TGM1* Exon 4 (gRNA3). gRNAs sequences are indicated within the colored boxes, with the protospacer adjacent motif (PAM) shown in bold. **c)** PCR analysis of genomic DNA from non-treated keratinocytes (NT) and Δ TGM1 keratinocytes treated with the RNP complex once (Δ TG1.1) or twice (Δ TG1.2), on both edited exons (e.g., E2 and E4). **d)** PCR analysis of genomic DNA from non-treated keratinocytes (NT) and Δ TGM1 keratinocytes treated with the RNP complex once (Δ TG1.1) or twice (Δ TG1.2), spanning either the Cas9 target sites to evaluate the excision of the entire genomic region of above 1.7 Kb between E2 and E4. The efficacy (densitometric) value of excision is indicated at the bottom of each lane. **e)** TIDE decomposition analysis of edited genomic sequences in Δ TGM1 keratinocytes with gRNA1/2 and gRNA3 showing the overall editing efficiencies and the percentage of indel formation (insertions and deletions) in both edited exons, compared to a wild-type sequence.

As expected from *TGM1* KO, TGM1 mRNA was totally absent in both the electroporated conditions (e.g., Δ TG1.1 and Δ TG1.2 keratinocytes) (**Figure 3.8. a**). Accordingly, western blot analysis demonstrated the absence of TG1 in Δ TG1 keratinocytes under differentiation conditions that stimulates its expression (**Figure 3.8. b**). Finally, the total protein extracts from Δ TG1 keratinocytes were tested for TG1 enzymatic activity, as compared to non-treated keratinocytes (**Figure 3.8. c**). Although Δ TG1.1 keratinocytes showed a minimum residual enzymatic activity (NT, 14d), the TG1 enzymatic activity was undetectable in Δ TG1.2 keratinocytes. Overall, these results demonstrate the feasibility and the high efficiency of *TGM1* knockout in human keratinocytes using the CRISPR-Cas9 system.

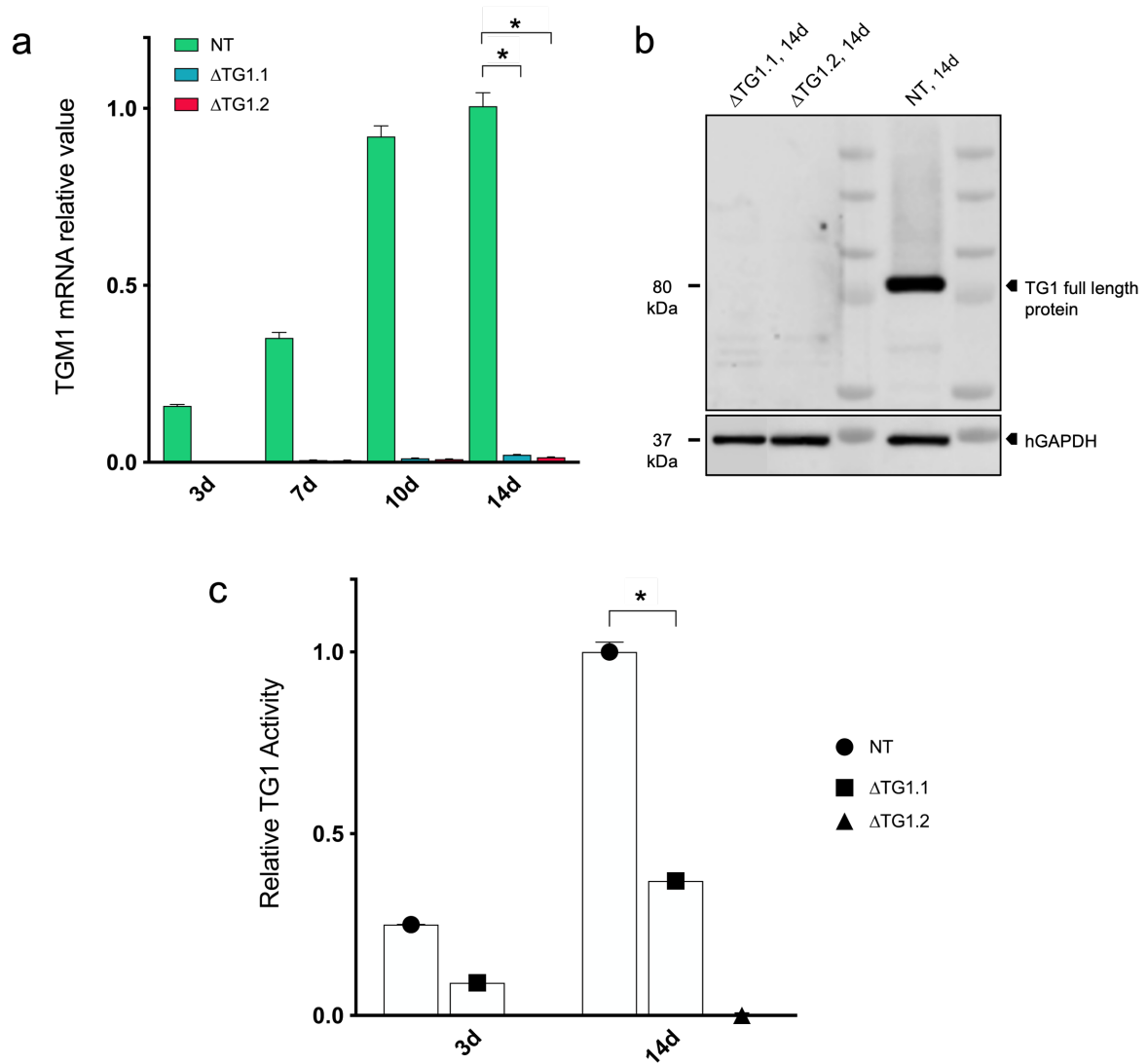


Figure 3.8. Total ablation of TGM1 mRNA and protein expression in Δ TGM1 keratinocytes. **a)** qRT-PCR quantification of the TGM1 mRNA relative expression in non-treated keratinocytes (NT), Δ TG1.1 and Δ TG1.2 keratinocytes, grown in standard culture medium for 3, 7, 10 or 14 days (3d, 7d, 10d, 14d). NT at 14d was arbitrarily set to 1. Expression levels were normalized per GAPDH. Statistical analysis: ANOVA test; asterisks indicate $p < 0.0001$. **b)** Western blot analysis of TG1 protein expression in non-treated (NT), Δ TG1.1 and Δ TG1.2 keratinocytes, grown in standard culture medium for 14 days (14d). 30 μ g of total protein extracts were loaded for each sample. **c)** Relative TG1 enzymatic activity evaluated in the same amount of total protein extract (50 μ g) from non-treated keratinocytes (NT), Δ TG1.1 and Δ TG1.2 keratinocytes, grown in standard culture medium for 3 and 14 days (3d, 14d). TG1 enzymatic activity of NT at 14d was arbitrarily set to 1. Statistical analysis: ANOVA test; asterisk indicates $p < 0.0001$.

3.3 Rescue of TG1 activity in Δ TGM1 cellular model: a proof of concept for LI gene therapy approach

Genetic correction of primary Δ TGM1 keratinocytes

Δ TGM1.2 keratinocytes (hereafter named Δ TGM1) were selected for proof-of-principle studies and preclinical evaluation of a gene therapy approach for Lamellar Ichthyosis (LI) (Figure 3.9).

Δ TGM1 keratinocytes were transduced with the SINy-RV-*TGM1* vector at MOI 2 using a RetroNectin®-bound method. Transduction efficiency was determined by the percentage of TG1 positive cells with immunofluorescent analysis and was approximately 50% (Figure 3.10).

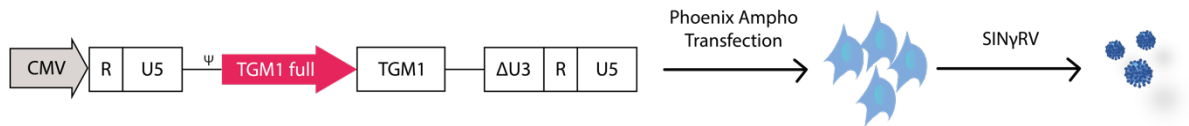


Figure 3.9. SINy-RV-*TGM1* vector. Schematic representation of transient production of self-inactivating retroviral vector (SINy-RV) carrying the endogenous *TGM1* full promoter (2.2 Kb) driving the expression of the *TGM1* coding sequence in Phoenix Ampho cell line.

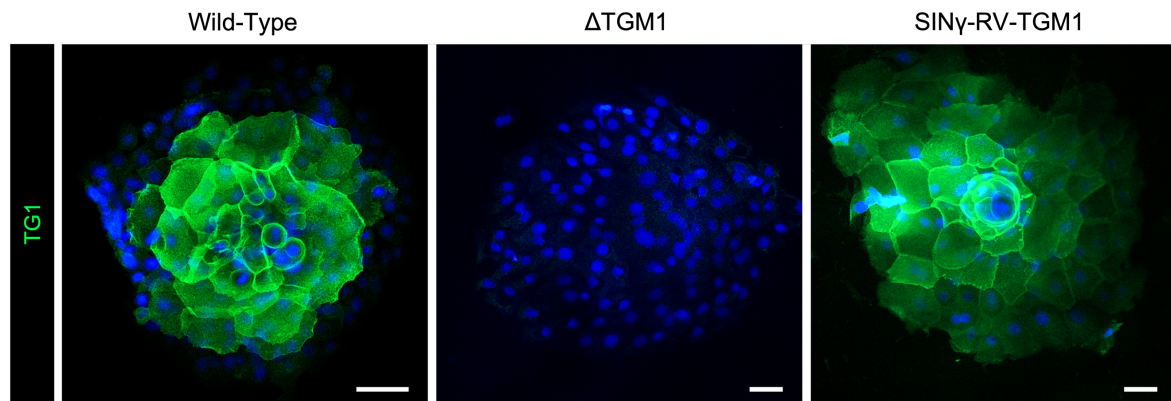


Figure 3.10. TG1 immunofluorescence staining in keratinocytes colonies. Representative images of immunofluorescence analysis at 7-day post plating of TG1 in colonies derived from wild-type, Δ TGM1, and SINy-RV-*TGM1*-transduced keratinocytes. Note that TG1 fluorescent signal is completely absent in Δ TGM1 keratinocytes. DAPI (blue) stains nuclei. Scale bars, 50 μ m

Formation of cross-linked envelopes

During the epidermal keratinization TG1 catalyzes the formation of the CEs, which are chemically resistant to ionic detergent and reducing agent, as described by H. Green in 1998 (Jeon et al. 1998). Since the cross-linking process is strictly related to the presence of TG1 in terminally differentiated keratinocytes, the absence of a functional protein should result in failure to form the cross-linked envelopes. Hence, the re-expression of TG1 should allow the restoration to a wild-type condition. Thus, SIN γ -RV-*TGM1*-transduced keratinocytes were analyzed for CE formation (**Figure 3.11**). Stratified cultures were exposed to 1 M Tris-HCl (pH 8.0), 2% 2-mercaptoethanol and 2% SDS for 10 minutes at 100°C, and cross-linked envelopes were isolated and counted. In Δ TGM1 keratinocytes, no typical envelopes were detected, and only few cell fragments were observed (**Figure 3.11. a**). Instead, we were able to isolate the typical polyhedral cross-linked envelopes both in wild type and in SIN γ -RV-*TGM1*-transduced keratinocytes (**Figure 3.11. a and b**). These preliminary results demonstrated that the exogenous TG1, produced in SIN γ -RV-transduced keratinocytes, restored TG1 enzymatic activity, thus it was able to catalyze the formation of CEs in stratified keratinocytes cultures.

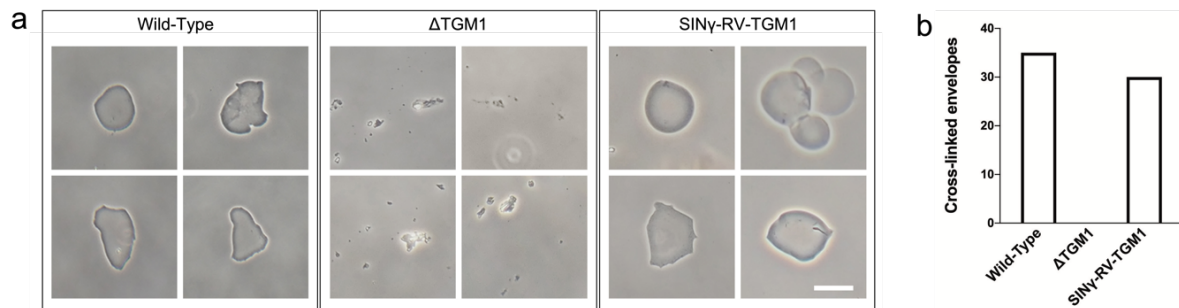


Figure 3.11. Isolation of cornified cell envelopes. **a)** Representative images of the cornified cell envelopes isolated from wild-type, Δ TGM1, and SIN γ -RV-*TGM1*-transduced keratinocytes after boiling cultures in 1 M Tris-HCl (pH 8.0), 2% 2-mercaptoethanol and 2% SDS for 10 minutes. Scale bar, 20 μ m. **b)** Numbers of cross-linked envelopes isolated from wild-type, Δ TGM1, and SIN γ -RV-*TGM1*-transduced keratinocytes normalized to the relative acquired fields under a phase contrast microscope.

3D skin equivalents with primary *TGM1*-corrected keratinocytes

We then seek to investigate the ability of genetically corrected *TGM1*/KO keratinocytes to induce the expression of properly localized and functional TG1 in a 3D skin equivalent model prepared with fibroblasts and wild-type, Δ TGM1, or SINy-RV-*TGM1*-transduced keratinocytes (**Figure 3.12**). Frozen sections of such 3D skin equivalents were immunostained and analyzed for TG1 expression and enzymatic activity (**Figure 3.12**). As shown in **Figure 3.12. c**, TG1 expression in SINy-RV-*TGM1*-transduced keratinocytes is normally restricted to the granular layer with the typical pericellular distribution, as compared to normal skin equivalents. Furthermore, the proper expression of exogenous TG1 in SINy-RV-*TGM1* transduced keratinocytes was associated to a full restoration of its enzymatic activity (Figure 3.12. **d**, **e**, and **f**).

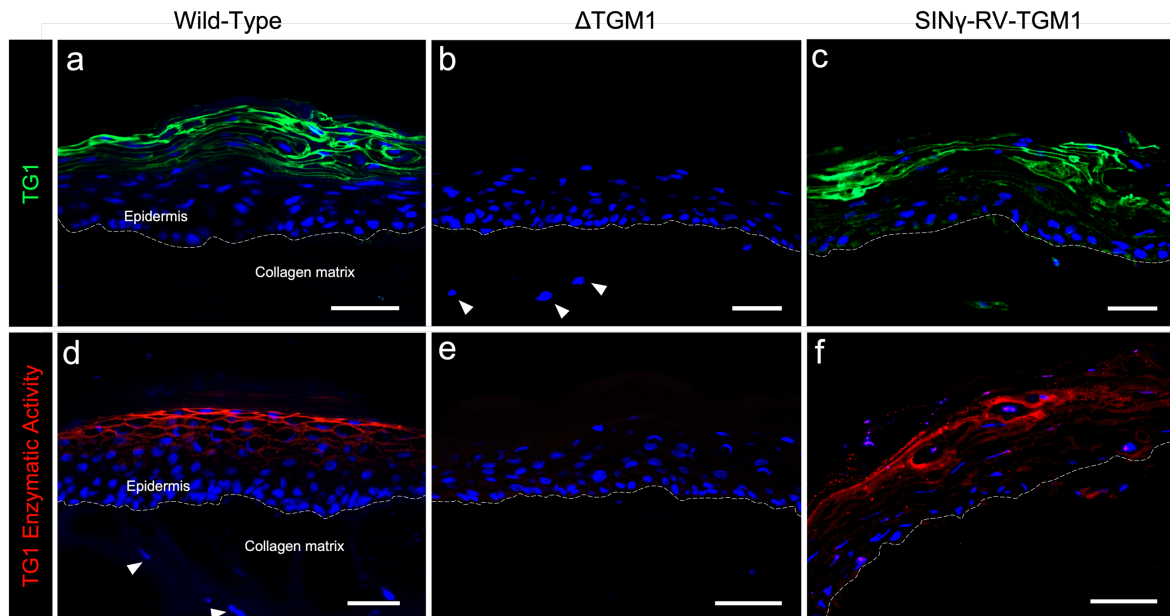


Figure 3.12. TG1 staining and functional activity in *TGM1*-corrected keratinocytes from organotypic 3D skin cultures. Collagen-based 3D skin equivalents were produced with wild-type keratinocytes, Δ TGM1 keratinocytes, or SINy-RV-*TGM1*-transduced keratinocytes. Immunostaining detection of TG1 (**a**, **b**, and **c**) and TG1 enzymatic activity (**d**, **e**, and **f**) reveal the normalized protein expression and recovered TG1 enzymatic activity in gene-corrected keratinocytes (SINy-RV-*TGM1*) evaluated with the incorporation of Alexa Fluor™ 594 Cadaverine. DAPI (blue) stains nuclei. White dotted line marks the epidermal-dermal junction. Arrowheads indicate the encapsulated fibroblasts within the collagen matrix. Scale bars, 50 μm.

Of note, the Δ TGM1 skin equivalent produced an epidermis characterized by a thickened stratum corneum (SC) strongly resembling the human ichthyosiform phenotype (**Figure 3.13. b**) (Chu 2008; Elias et al. 2010b; Arkin et al. 2015). In contrast, the SINy-RV-*TGM1* skin equivalent produced a normal SC, virtually indistinguishable to that observed in 3D skin equivalents generated by normal, wild-type keratinocytes (**Figure 3.13. a and c**). The presence and spatial distribution of TG1 substrates involucrin and LEKTI (Wiegmann et al. 2019), and the differentiation marker K10, were also assessed. Involucrin and LEKTI showed a diffuse distribution in Δ TGM1 skin equivalent (**Figure 3.13. e and h**), indicating the lack of protein cross-linking in the upper layers of epidermis, as typically occurs in LI (Hohl et al. 1993; Aufenvenne et al. 2012). In contrast, in SINy-RV-*TGM1* skin equivalent involucrin and LEKTI expression are correctly localized in the cellular periphery of the upper differentiated layers of the epidermis, resembling the wild-type condition (**Figure 3.13. d, f, g, and i**). The expression of K10, localized in the suprabasal cell layers, remained unchanged in all conditions (**Figure 3.13. j, k, and l**).

Altogether, these results demonstrated the restoration of TG1 expression using SINy-RV-*TGM1* in primary Δ TGM1 keratinocytes. In addition, these findings corroborate the valuable established tool for preclinical setting that recapitulates cellular hallmarks of the LI phenotypes *in vitro*.

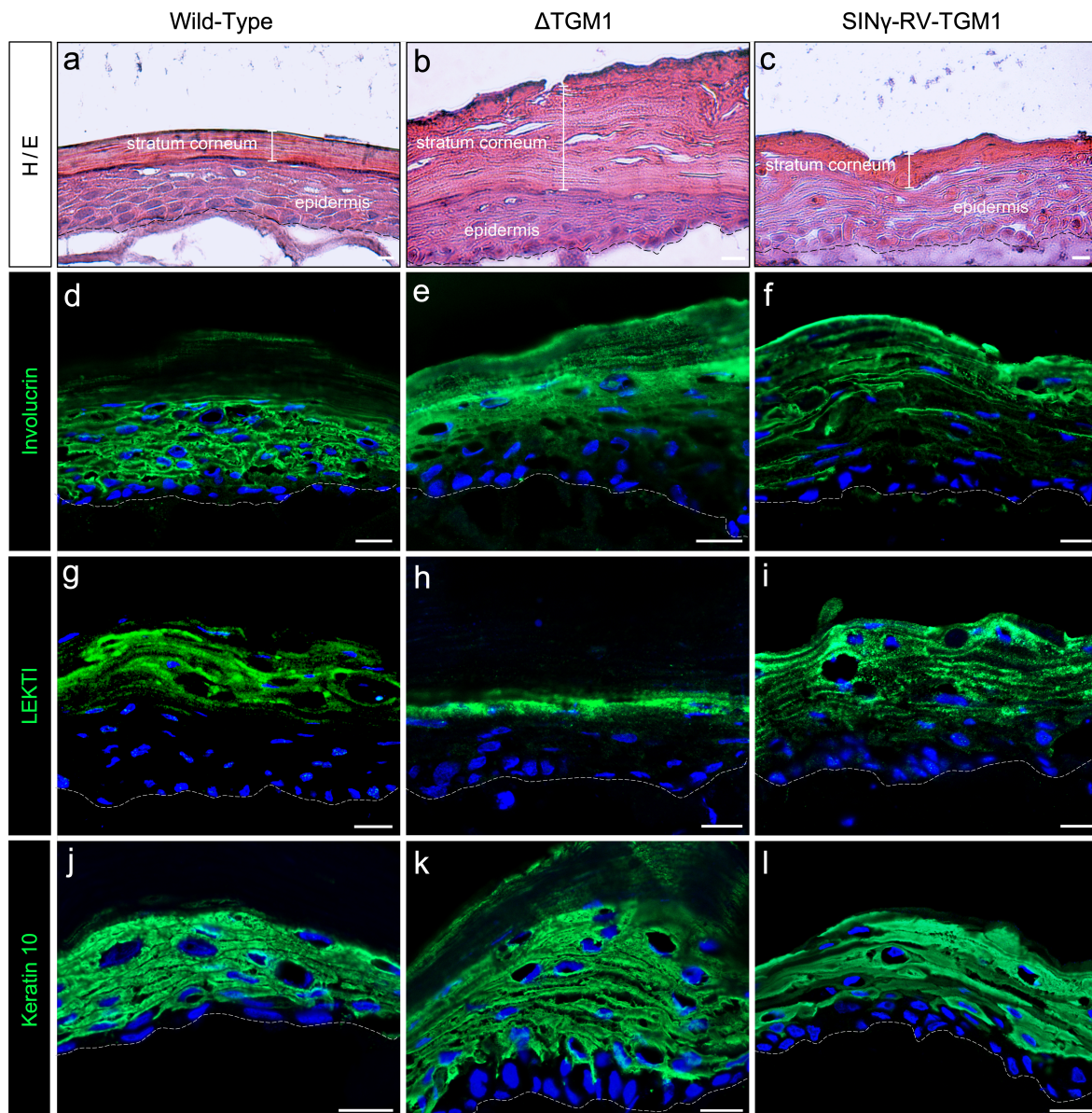


Figure 3.13. Characterization of organotypic 3D skin cultures. Collagen-based 3D skin equivalents were produced with wild-type keratinocytes, Δ TGM1 keratinocytes, or SIN γ -RV-TGM1-transduced keratinocytes. Histological analysis (H/E staining) shows the thickened stratum corneum in Δ TGM1 skin equivalents (b) in comparison with the normal epidermal architecture of wild-type (a) and SIN γ -RV-TGM1-transduced skin equivalents (c). Spatial distribution of TG1 substrates (Involucrin, LEKTI) and keratinocytes differentiation marker (Keratin 10) are shown. DAPI (blue) stains nuclei. White dotted line marks the epidermal-dermal junction. Scale bars, 20 μ m.

Discussion

TGM1-deficient Lamellar Ichthyosis is a rare, monogenic cornification disorder associated to severe clinical complications that highly decrease the patient's quality of life. It represents a life-long condition which greatly influences the appearance and physical state of the affected individuals. There is no cure for LI, and the existing treatments only aim to relieve the symptoms (DiGiovanna et al. 2013; Fleckman et al. 2013; Cortés et al. 2020). Thus, there is a significant unmet need for therapeutic strategies aimed at correcting the *TGM1* deficiency underlying LI.

Currently, the most innovative approaches (still not commercially available) are (i) enzyme replacement therapies (Aufenvenne et al. 2013; Plank et al. 2019) and (ii) the in vivo gene therapy strategy using a non-integrating herpes simplex virus type 1 vector, which express the human *TGM1* (Freedman et al. 2021). Although these therapeutical strategies are quite promising and technically feasible, and the latter is in Phase I-II clinical trial, they have a common drawback, namely they could likely have only a temporary beneficial effect. The epidermis is a highly renewing tissue, and the administered proteins or the non-integrating viral vectors have a limited and defined half-life. Thus, the *ex vivo* combined stem cell and gene therapy is the only feasible approach to permanently restore the epidermis of patients suffering from any devastating skin genetic disease. For instance, combined *ex vivo* cell and gene therapy of generalized intermediate LAMB3-Junctional Epidermolysis Bullosa (JEB) has proved to be life-saving and able to permanently regenerate a fully functional epidermis (up to 16 years follow-up) (Mavilio et al. 2006; Bauer et al. 2017; Hirsch et al. 2017; De Rosa et al. 2020; Kueckelhaus et al. 2021).

A first proof of concept of LI gene therapy was pioneered by Paul Khavari and his group in 1996, who published preliminary studies on the in vitro delivery of *TGM1* gene into LI patient cells using retroviruses (Choate et al. 1996a; Choate et al. 1996b). But this approach has never reached the clinic application.

Epithelial grafts from cultures of human clonogenic keratinocytes have been extensively used in many clinical settings with remarkable results both for cell therapy of severe burn patients and in cell and gene therapy approaches (Pellegrini et al. 1999; De Luca et al. 2006; Hirsch et al. 2017; De Luca et al. 2019). Recently it has been definitely demonstrated that the holoclone-forming cell is the only clonal type possessing self-renewing properties and long-term regenerative potential, being the stem cell of all squamous epithelia (Rama et al. 2010; Hirsch et al. 2017). The long-term restoration of a functional transgenic epidermis strictly relies on the genetic modification of long-lived, self-renewing epidermal stem cells, detected as holoclone-forming cells. In this light, ensuring the correction of the

entire stem cell population is one of the goals of this preliminary preclinical study that will be certainly evaluated in further experiments on LI patient-derived keratinocytes.

The gene therapy strategy implemented in this work exploits the use of a self-inactivating (SIN) integrating retroviral vector that represents a better and safer viral vector as compared to MLV- γ RV, which has been widely used for other clinical trials both in Europe and in USA. SIN vectors are characterized by a deletion of the U3 region of the 3' LTR, which eliminates a strong viral enhancer, thus reducing γ RV tendency to activate genes. But more importantly, an internal, often tissue-specific promoter of choice can be used to drive the transcription of the desired transgene (Yu et al. 1986; Kraunus et al. 2004). Therefore, the endogenous *TGM1* full promoter can be used to induce the physiological expression of TG1 in the upper epidermal layers. However, in these preliminary experimental settings keratinocytes transduction efficiency has never exceeded 50-60%. The cause is to be found in the inactivation of the 3' LTR that inevitably leads to low vector titers production. Furthermore, the transient production of SIN γ -RVs allowed extremely low vector production with titers of $\sim 10^6$ infectious particles/mL. Such hurdle will be exceeded, in the future, by the generation of a stable packaging cell line suitable for high titer SIN γ -RV large-scale productions and, of course, required along the path from bench to bedside. Nevertheless, stable packaging cell lines represent a very hard task and high time-consuming process, especially for SIN vectors, which necessarily needs the industry support (Maetzig et al. 2011). Our group, in collaboration with a Biotech company (BioNTech), produced a new clinically relevant SIN γ -RV carrying the COL7A1 gene allowing to reach almost a 100% of transduction efficacy in recessive dystrophic epidermolysis bullosa (RDEB) keratinocytes (our unpublished data), that will soon be used for a multicenter clinical trial. If the preliminary promising data will be confirmed on primary LI patient derived keratinocytes, the final goal will be to use this packaging cell line to produce SIN γ -RV-*TGM1* clinical grade viral supernatant to transfer this product in clinical settings.

This preliminary proof of principle study must necessarily be confirmed using LI patient cells from several donors with different genetic backgrounds in order to increase the study robustness. In addition, a significant increase in transduction efficiency is required to ensure the correction of the entire stem cell population. Such further studies also necessarily need the investigation of the effect of this therapeutic approach on holoclones (epidermal stem cell) and on keratinocytes clonogenicity since the efficacy of combined cell and gene therapy depends on the permanent engraftment of gene-corrected stem cells.

Altogether, the results obtained in this PhD thesis provided a prototype for the development of combined cell and gene therapy for *TGM1*-dependent LI, as a basis for future efforts to

allow the application of the proposed approach in Phase I/II clinical trials for the treatment of skin defect in LI patients.

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