Two Ancient Gene Families Are Critical for Maintenance of the Mammalian Skin Barrier in Postnatal Life

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The skin barrier is critical for mammalian survival in the terrestrial environment, affording protection against fluid loss, microbes, toxins, and UV exposure. Many genes indispensable for barrier formation in the embryo have been identified, but loss of these genes in adult mice does not induce barrier regression. We describe a complex regulatory network centered on two ancient gene families, the *grainyhead-like* (*Grhl*) transcription factors and the protein cross-linking enzymes (tissue *transglutaminases* [*Tgm*s]), which are essential for skin permeability barrier maintenance in adult mice. Embryonic deletion of *Grhl3* induces loss of *Tgm1* expression, which disrupts the cornified envelope, thus preventing permeability barrier formation leading to neonatal death. However, gene deletion of *Grhl3* in adult mice does not disrupt the preformed barrier, with cornified envelope integrity maintained by *Grhl1* and *Tgm5*, which are up-regulated in response to postnatal loss of *Grhl3*. Concomitant deletion of both *Grhl* factors in adult mice induced loss of *Tgm1* and *Tgm5* expression, perturbation of the cornified envelope, and complete permeability barrier regression that was incompatible with life. These findings define the molecular safeguards for barrier function that accompany the transition from intrauterine to terrestrial life.

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INTRODUCTION

The mammalian skin, the largest of all the organ systems, forms a critical interface between the highly regulated internal milieu and the challenging external environment. The outermost layer of the skin, the epidermis, is essential for the establishment, maintenance, and repair of the barrier function, which is achieved through the orchestrated regulation of structural and junctional proteins, lipids, enzymes, and transcription factors (Natsuga, 2014; Roop, 1995; Segre, 2003). This barrier function is critical as a defense against excess permeability, UV light exposure, microorganisms, and other environmental insults. The most superficial layer of the epidermis, the stratum corneum, forms the front line of the barrier (Candi et al., 2005). In this layer, differentiation-associated keratin intermediate filaments form a complex scaffold that fills the keratinocytes, providing mechanical strength. The periphery of the cells is lined with a membranous structure, the cornified envelope (CE), which is composed of multiple proteins including involucrin, loricrin, envoplakin, periplakin, small proline-rich proteins, keratin intermediate filaments and others, all cross-linked by transglutaminases (Tgms), a family of calcium-dependent enzymes critical for the integrity of the CE and barrier function. Intercellular spaces filled with lipid-enriched lamellar membranes complete the watertight seal (Candi et al., 2005; Marshall et al., 2001; Natsuga, 2014; Proksch et al., 2008). The CE thus provides an important scaffold for the lipid membranes that contribute to the permeability barrier (Elias et al., 2002; Schmuth et al., 2004).

Many insights into mammalian barrier establishment have come from naturally occurring and genetically engineered mouse models (Natsuga, 2014). Our laboratory has contributed to this through the identification and characterization of the grainyhead-like (*Grhl*) gene family, which encodes transcription factors that regulate the formation and maintenance of the integument in diverse species across 700 million years of evolution (Jane et al., 2005; Ting et al., 2003b; Wilanowski et al., 2002). The three mammalian members (*Grhl1-3*) demonstrate remarkable conservation of sequence, expression, and function with the antecedent *Drosophila* gene, *grainyhead* (*grh*). The *grh* mutants exhibit a range of integument phenotypes (Lee and Adler, 2004; Mace et al., 2005; Ostrowski et al., 2002), which are mirrored in *Grhl* mouse mutants (Caddy et al., 2010; Ting et al., 2003a; Wilanowski et al., 2008). *Grhl1*-deficient mice display impaired cellular adhesion manifesting as defective hair anchoring and

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Abbreviations: CE, cornified envelope; ChIP, chromatin immunoprecipitation; E, embryonic day; Grhl, grainyhead-like; K, keratin; TEWL, transepidermal water loss; Tgm, transglutaminase; TSLP, thymic stromal lymphopoietin

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palmo-plantar keratoderma (Wilanowski et al., 2008) but have no overt skin barrier defect at birth. They have normal transepidermal water loss (TEWL) and toluidine blue dye exclusion assays, and normal levels of thymic stromal lymphopoietin (TSLP) and the antimicrobial peptides S100A8 and S100A9, all biomarkers of barrier integrity (Kypriotou et al., 2012; Ziegler et al., 2013). In later life, they display a mild elevation of TSLP, S100A8, and S100A9 levels only, possibly indicative of subclinical barrier dysfunction (Mlacki et al., 2014). In contrast, *Grhl3*-null mice ($Grhl3^{-/-}$) fail to form any functional skin barrier during embryogenesis and die immediately postnatally from excessive water loss (Ting et al., 2005a). Loss of expression of *Tgm1*, a direct target of GRHL3, is thought to contribute to this defect (Hopkin et al., 2012; Ting et al., 2005a), as mice lacking *Tgm1* also die immediately postnatally with no barrier function (Matsuki et al., 1998). Altered expression of other structural proteins, enzymes, and lipids of the CE are also observed in the *Grhl3*-null mice and presumably contribute to the barrier defect (Ting et al., 2005b; Yu et al., 2006), but it is yet to be established which of these are direct *Grhl3* targets and which are altered in response to loss of barrier function.

Despite its developmental importance, conditional loss of *Grhl3* expression in the neonatal epidermis after barrier formation is complete is not associated with overt barrier regression, and the mice survive for >12 months before succumbing to squamous cell carcinomas (Darido et al., 2011). Skin barrier preservation occurs despite changes in expression of filaggrin, involucrin, and the keratins K5, K6, and K10 in the *Grhl3* conditional knockout epidermis that are similar to those seen in epidermis from $Grhl3^{-/-}$ mice that lack any barrier function (Darido et al., 2011). These findings suggest that compensatory factors exist in *Grhl3*-deficient adult mice that maintain the epidermal barrier. In this study we used the *Grhl3* conditional knockout mice to define a complex regulatory network composed of *Grhl* factors and *Tgm* family members that is critical for maintenance of the epidermal barrier in adult life.

RESULTS

Assessment of skin permeability barrier function in adult Grhl3-deficient mice

Grhl3 conditional knockout mice ($Grhl3^{\Delta/-}K14Cre^{+}$) (Darido et al., 2011) display no overt barrier defect, with 100% survival at weaning (Figure 1a). Analyses of TEWL (Figure 1b), toluidine blue dye penetration (Figure 1c), or *TSLP, S100A8,* and *S100A9* expression (Figure 1d) at 6 weeks of age are consistent normal barrier function. Expression of *Tgm1*, a key *Grhl3* target for barrier establishment in utero (Hopkin et al., 2012; Ting et al., 2005a), was significantly reduced in both $Grhl3^{-/-}$ and $Grhl3^{\Delta/-}K14Cre^{+}$ mice compared to controls (Figure 1e). Despite this finding, the morphological appearance of the CE was completely different between these mutant lines; $Grhl3^{-/-}$ embryos showed major disruption, whereas those from $Grhl3^{\Delta/-}K14Cre^{+}$ mice were indistinguishable from wild type (Figure 1f). Exposure of the CE to ultrasound for different time periods (a measure of their fragility) emphasized this difference (see Supplementary Figure S1 online). These findings suggest that preservation of the functional skin barrier in the $Grhl3^{\Delta/-}K14Cre^{+}$ adult mice was due to a factor that could maintain CE integrity in the context of reduced *Tgm1* expression, presumably another member of the *Tgm* family. Consistent with this finding, the Tgm activity in the $Grhl3^{\Delta/-}K14Cre^{+}$ mice was comparable to that of wild-type controls (Figure 1g).

Tgm5 expression is up-regulated in $Grhl3^{\Delta/-}K14Cre^{+}$ skin in response to increased Grhl1

In addition to *Tgm1*, both *Tgm3* and *Tgm5* have been implicated in cornification of the upper epidermis (Candi et al., 2001; Kalinin et al., 2001). We examined the expression of these enzymes in the $Grhl3^{\Delta/-}K14Cre^{+}$ epidermis to determine whether a compensatory increase was evident in response to loss of *Tgm1*. *Tgm3* was not altered between wild-type and mutant mice (Figure 2a), but *Tgm5* was substantially increased at 6 weeks (Figure 2b), a finding that was not observed in the epidermis of embryonic day (E)18.5 $Grhl3^{-/-}$ embryos (Figure 2c). The DNA consensus-binding site for the human and *Drosophila* GRHL factors (AACCGGTT) has been conserved across 700 million years (Ting et al., 2005a), and we have successfully used a phylogenetic approach to identify GRHL binding motifs in regulatory sequences conserved across all placental mammals (Caddy et al., 2010; Darido et al., 2011). Examination of the *Tgm5* regulatory regions identified a highly conserved GRHL site in the proximal promoter (Figure 2d), and chromatin immunoprecipitation (ChIP) defined this as a binding site predominantly for GRHL1 (Figure 2e). Consistent with *Tgm5* being a physiological target of GRHL1, expression was reduced in the skin of $Grhl1^{-/-}$ mice (Figure 2f), and the level of *Grhl1* expression was increased in the skin from $Grhl3^{\Delta/-}K14Cre^{+}$ mice (Figure 2g) but not in E18.5 $Grhl3^{-/-}$ embryos (Figure 2h), mirroring the pattern of *Tgm5* expression.

Assessment of barrier function in $Tgm5^{-/-}$ mice

To assess the role of *Tgm5* in skin barrier function, we generated a $Tgm5^{-/-}$ line using standard gene targeting approaches (see Supplementary Figure S2a–c online). $Tgm5^{-/-}$ mice were present in normal Mendelian ratios at weaning (Figure 3a), despite complete lack of *Tgm5* expression at both the RNA (Figure 3b) and protein levels (Figure 3b, inset). The animals exhibited no evidence of barrier dysfunction at E18.5, no increase in TEWL (Figure 3c), and normal toluidine blue dye exclusion (see Supplementary Figure S2d). This latter assay was also normal in 6-week-old mice (Figure 3d), as were the levels of *TSLP, S100A8,* and *S100A9* (Figure 3e) and CE integrity (Figure 3f). Humans carrying mutations in the *TGM5* gene exhibit acral peeling skin syndrome (Cassidy et al., 2005), which manifests as superficial blistering and peeling on the volar and dorsal aspects of the hands and feet associated with increased loricrin expression (Pigors et al., 2012). Compensatory increases in TGM1 or TGM3 are not seen in these patients (Pigors et al., 2012). $Tgm5^{-/-}$ mice exhibited no evidence of skin peeling on any surface, and epidermal loricrin RNA and protein levels were unchanged compared with WT controls (see Supplementary Figure S2e). However, expression of *Tgm1* was markedly increased (Figure 3g) in response to an almost three-fold increase in expression of *Grhl3* (Figure 3h). *Tgm3* expression was not altered (see Supplementary Figure S2g).