

**TRANSGLUTAMINASE 1 REPLACEMENT THERAPY SUCCESSFULLY
MITIGATES THE ARCI PHENOTYPE IN FULL-THICKNESS SKIN DISEASE
EQUIVALENTS**

SHORT TITLE: SUCCESSFUL TOPICAL TRANSGLUTAMINASE 1 REPLACEMENT

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Abbreviations:

ARCI, autosomal recessive congenital ichthyosis; dPG, dendritic polyglycerol; Da, Dalton; NHEK, normal human epidermal keratinocytes; P_{app} , apparent permeation coefficient; PBS, phosphate buffered saline; pNIPAM, poly(N-Isopropylacrylamide); SC, stratum corneum; TEWL, transepidermal water loss; TG1, transglutaminase 1; tNG, thermoresponsive nanogels; VPTT, volume phase transition temperature

Letter to the Editor

Autosomal recessive congenital ichthyosis (ARCI) disrupts normal keratinization resulting in generalized scaling of the skin. There are presently no curative therapies available (Fleckman et al. 2013), local protein replacement is therefore an encouraging approach for a more specific treatment. ARCI refers to a heterogeneous group of rare skin keratinization disorders with an estimated prevalence of 1 in 50,000 – 200,000 (Dreyfus et al. 2014). The disease is characterized by notable impairments to the skin's barrier-function, resulting in frequent infections and increased transepidermal water loss (TEWL). ARCI are caused by mutations in one of twelve identified genes involved in epidermal differentiation, the most common of which are loss of function mutations in *TGMI* that affect ~30% of patients (Rodriguez-Pazos et al. 2009). *TGMI* encodes transglutaminase 1 (TG1), a protein that plays an essential role in the formation of the cornified envelope (Eckert et al. 2005). Since animal models of severe keratinization disorders such as ARCI are not viable and animal skin is a poor representation of human skin (Gerber et al. 2014), the use of organotypic skin equivalents has emerged as a valid tool to investigate ARCI. In the present study, full thickness skin equivalents were generated from fibroblasts and keratinocytes of ARCI patients with mutations in *TGMI* and topically treated with TG1. Since biomacromolecules do not normally overcome the skin-barrier owing to their high molecular weight, protein delivery was mediated by loading onto thermoresponsive nanogels (tNG) (Cuggino et al. 2011). tNGs have been shown to encapsulate proteins as large as 150 kDa, and to then release these upon thermal trigger (Giulbudagian et al. 2018b; Witting et al. 2015). Our groups previously reported the epidermal delivery of functional TG1 using tNGs to rescue the barrier defects of *TGMI* knockdown skin equivalents (Witting et al. 2015). The transient nature of induced gene knockdowns, however, limits their usefulness. Additionally, it remains unclear whether TG1 loaded tNGs is an effective topical treatment for ARCI patients with *TGMI* mutations.

To address this, full-thickness skin equivalents were generated from fibroblasts plus normal keratinocytes, keratinocytes with transient *TGMI* knockdowns, or keratinocytes from ARCI patients with *TGMI* mutations (Fig. 1). In comparison to normal equivalents, *TGMI* knockdown and Patient equivalents both demonstrated a slightly thinned SC and epidermis and a reduced cell number within the granular layer. The epidermal differentiation markers keratin 14 and 10 were appropriately distributed. TG1 activity was present in normal skin equivalents but not in those generated from patient cells or *TGMI* knockdown keratinocytes, in line with the inactivating mutations found in Patient 1 and the absence of persistent TG1 expression in Patient 2 and knockdown equivalents. Notably, decreasing efficiency of *TGMI* repression in knockdown equivalents was observed with transcript levels recovery to >50% after 10 days cultivation (Fig S1).

To assess the biocompatibility of TG1 loaded tNGs, normal keratinocytes and fibroblast monolayers and keratinocytes derived from Patients 1 and 2 were incubated with TG1 loaded tNGs for up to 48 h resulting in no significant cytotoxicity at any of the tested concentrations (Fig. 2, S2, S3). Concordantly, no significant cytotoxicity was observed following the application of tNGs onto skin equivalents (Fig. 2c). Additionally, the ability of TG1, either alone or loaded in tNGs, to enter keratinocytes was assessed by confocal microscopy. TG1 entered the cytoplasm in a time-dependent manner, whereas tNGs were taken up more rapidly (Fig. S4). This and the lack of clear intracellular co-localization would suggest that the tNGs and TG1 enter keratinocytes separately, in line with the relatively quick release of protein at temperatures $\geq 35^{\circ}\text{C}$. Overall, however, tNGs coming into direct contact with cells of the viable epidermis is rather unlikely since there is evidence to show tNG are largely unable of overcoming the SC even of barrier deficient skin (Giulbudagian et al. 2018a).

Finally, Patient 1 skin equivalents were treated topically with TG1 in solution or loaded in tNGs four times over eight days. Untreated Patient 1 equivalents demonstrated decreased

barrier function as shown by the significant increases in their apparent permeabilities (P_{app}) to testosterone as compared to normal equivalents (Fig. 2a). Following full treatment regimens with TG1-loaded tNGs, a significant decrease in P_{app} – indicating improved barrier function – was seen in a TG1 dose-dependent manner (Fig. 2a, d, S5). Activity staining confirmed the delivery of functional TG1 into viable epidermal layers (Fig. 2e) and the distribution of activity was comparable to normal equivalents. Improvement of barrier activity was confirmed by permeability tests with Lucifer Yellow (Fig. 2f) and N-hydroxy-sulfosuccinimide-LC-biotin (Fig. S6). The normalized amount of Lucifer Yellow fully passing the skin model was increased by more than 8-fold in patient models compared to control models, and reduced to 116% compared to controls following treatment with TG1-loaded tNGs, indicating a major restoration of the barrier function. Importantly, P_{app} was unaffected by the application of unloaded tNG or TG1 dissolved in PBS only (Fig. 2a, b). It is highly likely that the majority of TG1 penetrating into the viable epidermis did so independently of the tNGs since they do not overcome the stratum corneum (Giulbudagian et al. 2018a).

The aim of this study was to further characterize the therapeutic potential of TG1 loaded tNGs in ARCI skin, as well as to better understand their mechanism of action based on a previously demonstrated proof-of-principle of epidermal delivery of TG1 by topical application of TG1 loaded tNGs (Witting et al. 2015). Overall, these data verify that topical protein substitution could mitigate or even reverse the ARCI disease phenotype. Notably, Traupe and colleagues previously demonstrated that topical applications of TG1 mixed with cationic liposomes successfully delivered the functional protein to skin equivalents made from *TGMI* mutant ARCI patient cells, grafted onto humanized mice (Aufvenne et al. 2013). In contrast to our system, no changes to barrier function were observed upon treatment, likely a result of their model; grafted animals demonstrate compact hyperkeratosis unlike the typical skin-barrier ARCI phenotype, and showed transepidermal water loss close to normal levels.

In summary, topical TG1 replacement therapy is a highly promising therapeutic avenue for ARCI patients with disease-causing *TGMI* mutations. The work here indicates TG1 delivery to the intercellular spaces between keratinocytes, and possibly their intracellular environments, can produce therapeutic improvements to the skin-barrier function of the ARCI phenotype. It is hypothesised that increasing the concentration or enzymatic activity of TG1 within the tNG will result in improved therapeutic efficacy and is the likely starting point for future development. The ability of tNGs to encapsulate a wide variety of proteins and deliver these past the SC of barrier deficient skin makes them a promising platform technology to treat a range of inflammatory and monogenic skin diseases.

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Figure Legends

Figure 1, Characterization of full-thickness skin equivalents: (a) cryosections of (i) normal, (ii) *TGMI* knockdown, (iii) ARCI Patient 1 and (iv) ARCI Patient 2 skin equivalents. From left to right, images show H&E, keratin 14 (green), keratin 10 (green), transglutaminase 1 (TG1; green), and TG1 activity staining (counter-staining with DAPI in blue). Scale bars = 50 μm . The dashed, yellow line indicates the epidermal – dermal junction. (b) The viability of normal human keratinocytes derived from healthy subject (black), Patient 1 (light grey) or Patient 2 (dark grey) following (i) 24 h or (ii) 48 h incubation with TG1, tNGs and TG1 loaded tNGs as assessed by MTT assay. (iii) Viability of normal skin equivalents following full treatment regimen with TG1 loaded tNGs (5 $\mu\text{g}/\text{cm}^2$ TG1, 500 $\mu\text{g}/\text{cm}^2$ tNG; applied 4 times over 8 days) as assessed by MTT assay; results are expressed as % untreated control. Statistical differences were assessed by one-way ANOVA with Dunnet's correction for multiple comparisons ($n = 3$). Sodium dodecyl sulphate served as positive control, respectively.

Figure 2, Skin-barrier function and transglutaminase 1 (TG1) activity of skin equivalents following TG1-loaded thermoresponsive nanogels (tNG) treatment: (a) Apparent permeabilities (P_{app}) of normal (white) or Patient 1 (gray shades) skin equivalents, untreated (UT) or following treatment with TG1 in phosphate buffered saline (PBS) or tNGs ($n = 3$, error bars = SEM, * $p \leq 0.05$, ** $p \leq 0.01$). Corresponding permeation profiles of skin equivalents following treatment with (b) unloaded tNG, (c) TG1 in PBS or (d) TG1 loaded tNGs. The untreated controls (UT) are identical in all panels. (e) TG1 staining and activity in (i) Patient 1 model treated with TG1/PBS (control), (ii) normal model treated with TG1/tNG, (iii) TG1 knockdown model treated with TG1/tNG and (iv) Patient 1 model treated with TG1/tNG (blue

= DAPI, green = TG1 and biotinylated-cadaverine staining, respectively; scale bars = 50 μm).

(f) Lucifer Yellow permeability in (i) normal skin model, (ii) Patient 1 skin model, and (iii)

Patient 1 skin model treated with TG1/tNG. The dashed, yellow line indicates the epidermal-

dermal junction (blue = DAPI, green = Lucifer Yellow; scale bars = 75 μm).



